CRANFIELD UNIVERSITY

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"DEVELOPMENT OF ELECTROCHEMICAL SENSORS FOR THE DETECTION OF PHOTOSYSTEM INHIBITING HERBICIDES"

CRANFIELD CENTRE FOR SUPRAMOLECULAR TECHNOLOGY (CCST)

INSTITUTE OF BIOSCIENCE AND TECHNOLOGY

PhD Thesis

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SUPERVISED BY PROF. SERGEY PILETSKY

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This thesis is submitted in partial fulfilment of the requirement for the degree of Doctor of Philosophy

To life’s little miracles. To the people that come and go, my special moments with them, and the person that lingers with me.

“When you really want something to happen, the whole universe conspires so that your wish comes true”. “Those who do not have the courage to follow their “Personal Myth” are doomed to a life of emptiness, misery, and unfulfillment”.

Paulo Coelho “The Alchemist”

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ABSTRACT

The objective of the present work is the development of an amperometric sensor for detection of hydrogen peroxide and its integration with spinach chloroplasts for the further development of a sensor for herbicides. The design of the sensor employed screen-printing electrodes which are easily produced at the facilities available in Cranfield University. The hydrogen peroxide sensor has been based on horseradish peroxidase (HRP) as the catalytic element and hydroquinone as the mediator. HRP has been immobilised onto the sensor surface using a newly developed thioacetale-based polymer capable of covalent immobilisation of primary amines. A new HRP-based biosensor was screen-printed using a carbon/polymer mixture. Hydrogen peroxide concentrations were analysed at the reduction potential of hydroquinone (–0.3 V). The biosensors developed in this work had low detection limit of H_2O_2 (0.1 μM), long term stability (they can be stored for 2 months at 4 ^{0}C) and good reproducibility of measurements (RSD ~ 5%).

The hydrogen peroxide sensor has been further integrated with spinach chloroplasts in an attempt to create a sensor for photosynthesis-inhibiting herbicides. It was found however that the quantity of H_2O_2 generated by chloroplasts in our experimental conditions was not sufficient to allow quantitative analysis. Due to this we have developed an alternative approach based on the electrochemistry of the Hill reaction. In this reaction the photosynthetic process and electron flow passing through photosystem II (PSII) is monitored through the quantity of reduced artificial electron acceptor. Upon illumination of the chloroplasts a signal from a reduced acceptor or mediator was recorded chronoamperometrically. The added herbicide inhibits the photosynthetic process and decreases the reduction of mediator. The decrease in measured current which is proportional to herbicide concentrations have been used for herbicide detection. Three mediators of Hill reaction were tested including 2,6 dichlorophenolindophenol (DCPIP) duroquinone and potassium ferricyanide. The optimal results were obtained using DCPIP. The optimal wavelength for the excitation of chloroplasts was 650 nm. The chloroplasts have been immobilised onto the sensor surface using cross-linking with glutaraldehyde and bovine serum albumin. The developed system allowed reliable detection of herbicides (RSD = 10%) with a detection limit of 1-8 nM depending on the type of herbicide. The sensor can be stored for 3 months at -80 ^{0}C. Preliminary measurements of river water samples using this sensor were also performed indicating good correlation between the data obtained with GC-MS and the chloroplast-based biosensor developed in this study.

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CHAPTER 1. GENERAL INTRODUCTION

1.1 AIM AND OBJECTIVES

This work is part of a European project known as BEEP (Biosensors for Effective Environmental Protection) which is aimed at the assembly and application of Photosystem II-based biosensors for large scale environmental screening of specific herbicides and heavy metals.

Photosystem II is a protein complex located in the thylakoid membrane of algae, cyanobacteria and higher plants such as spinach. In the presence of artificial acceptors, the electron transfer through the isolated PS-II particles and the accompanied oxygen release could be inhibited by herbicides or heavy metals. The inhibition of the photosynthetic activity of PS-II can be measured by the means of oxygen evolution, electron transfer or fluorescence and could be further translated by either amperometric or optical systems. This opens a possibility for developing sensor systems for detection of herbicides/heavy metals at nanomolar-picomolar concentration range.

The objective of the present work is development of an amperometric sensor for detection of hydrogen peroxide and its integration with PSII for the further development of sensor for herbicides. The design of the sensor employed screen-printing electrodes which are easily produced at the facilities available in Cranfield University or bought from external sources. This screen-printing technology is well-known procedure for a large-scale production of electronic components and sensors. Preliminary measurements involved the detection of hydrogen peroxide using horseradish peroxidase as the suitable enzyme and hydroquinone as the mediator. Several types of electrodes were tested in order to evaluate the best which will be later on integrated with isolated PS-II. In order to achieve that, the immobilised PS-II must be stable and capable of regeneration after interaction with herbicides and heavy metals. Electrodes were further tested with a flow-injection system and a portable device will be produced for the purpose of the whole project.

1.2 ENVIRONMENTAL MONITORING

As industry started to evolve, life requirements became more and more demanding in terms of goods provided. The mass production of them has led to the discovery of new materials and in particular chemicals that have influenced humans in all life aspects. Thus a large scale use of chemicals such as pesticides in agriculture and forestry management affects public health. The pesticides introduced in practice since 1945, have spread worldwide as the most significant form of pest control. Their availability on the commercial market reaches a point of thousands of different compounds including herbicides, bactericides, insecticides and fungicides. Herbicides are considered to be one of the most important classes of pesticides used in European Union.

In recent years, concerns have risen about the side effects of these compounds due to their intentional or accidental release in the environment. After the industrial revolution and the increase in chemical technologies, concerns for minimisation of pollutants and alternative "clean technologies", have become the common EU strategy. The whole control process started with tighter environmental legislation that were introduced in order to minimise the release of harmful pollutants either having immediate affects or long term ones. As a consequence from the chemical release, there is an increase of contaminated sites that need to be sampled (Grasselli, 1992). The medium that interests researchers inside and outside EU countries focuses on drinking water, air, groundwater, river water, seawater and soil. Different methods are usually required for the preparation of samples from such matrices, including extraction techniques and analysis.

Environmental mode of analysis includes both *in-situ* and *ex-situ* techniques. The *ex-situ* analysis requires sampling and transportation of samples to the laboratory for detailed measurements. In this case, skilled personnel are required for an elaborate sample pre treatment using sophisticated and expensive equipment. The data from a laboratory analysis could be more accurate and could provide lower detection limits than *in-situ* ones. In *in-situ* analysis the data can be collected and analysed at the selected site of interest immediately by an instrument (possibly automatic), which is easy to handle. The low cost and the speed of *in-situ* analysis has made this technique desirable. In order to analyse data of river water, seawater or even soil, portable equipment is required which is relatively inexpensive and easy to handle. Using *in-situ* analysis could provide enough

information required in order to minimise the samples transported to the laboratory for a more precise assay. Pre-screening of samples could reduce the sample load and give an indication of the contamination that would also help to use the right laboratory technique.

For all the above mentioned reasons, the development of equipment for a quick *in-situ* analysis is of high importance. Furthermore for the environmental monitoring *in-situ* instrumentation is essential for keeping track of changes in the environment in a short period of time in order to assist the industry to identify pollution sources and plan preventive and recuperation actions. Existing techniques need to be improved in terms of sensitivity and stability. The use of biosensors could provide a useful analytical tool in the environmental sector as well as already has done for the medical one, with the greatest example being the glucose sensor (Turner, 2001).

1.3 EU STANDARDS

For several decades now, an increase in the food requirements and production has led to a common objective throughout Europe. Agriculture industry has being constantly raising their outputs ever since 1940s in order to respond to such objectives. In 1980, Drinking Water Directive (80/778/EEC) has focussed on the impact of diffuse agriculture pollution of drinking water. Authorities such as the U.S. Environmental Protection Agency (EPA), EU Water Framework Directive and the UK Department of the Environment have introduced legislation defining maximum concentrations of agrochemicals in drinking water (Nicholson and Blaine, 1993). Many pesticides are among the list of dangerous pollutants – the subject of this legislation. Information for these compounds (targets, instructions for application, legislation) can be found in "The Pesticide Manual" (Tomlin, 2003). EU Directive 91/414/EEC regulates the authorisation of the materials, their placement in the market and the monitoring of plant protection products in the European Community. From the 99 active substances which are included in the first stage of the EU working program, the 33 of them are herbicides. In 1993, the total number of pesticides available for use in the European Community countries was 808. Their placement in the market is regulated by directive 98/8/EC. In 2000, the introduction of the new Water Framework Directive (WFD) has made changes that incorporate frameworks for assessment, monitoring, and management of surface and ground waters based on their chemical and ecological status. Finally in 2002, the

Commission proposed several criteria for assessing the chemical status of all the pollutants. The present limit value for active substances contained in pesticides and their metabolites is in the area of 0.1 μg/l which is a limit only for authorisation properties.

In 1992, and during the 5th EU Environmental Action Programme "Towards Sustainability", the requirements for a broad instrument use for pollution control have being introduced.

1.4 WATER ANALYSIS

Water analysis is a complex process since it is a medium where micro-organisms and animals live and reproduce, changing constantly the chemical content of the water. In addition, the changes to the hydrological cycle could also affect and imbalance the system making its monitoring necessarily. Factors such rainfall, precipitation, evaporation and river runoff could influence and change the water consistency and composition. Any damage to wildlife and treat to human life could be controlled by water measurements in order to pinpoint the cause as well as the source. For example analysis of wastewater plants could monitor the effectiveness of the treatment processes. When dealing with water samples, one should know and take into account the difficulties inherent in this matrix.

According to Water Act 2003 (UK chapter 37) wastewater dischargers require permits and according to these permits the limits are set on the amounts of specific pollutants which can be discharged, as well as a schedule for monitoring and reporting the results. Only standard analytical procedures could be used providing assurance to government agencies that the results from different laboratories are comparable and well reproduced. Generally in order to collect the water samples it is necessary first to assess the site where collection would be undertaken and set a limit of samples that would give sufficient results. Once representative samples are gathered, the next step would be extraction of the analyte from the bulk solution, since most of the analytical methods require the analyte to be in the liquid phase. Some measurements even require separating the analyte from the water entirely. Depending of the solubility of the analyte, the separation solvent could be either organic or a mixture of both organic and inorganic

ones. A more detailed water analysis is illustrated in Figure 1.1, and includes the areas where biosensors could be added to provide a more efficient analytical tool.

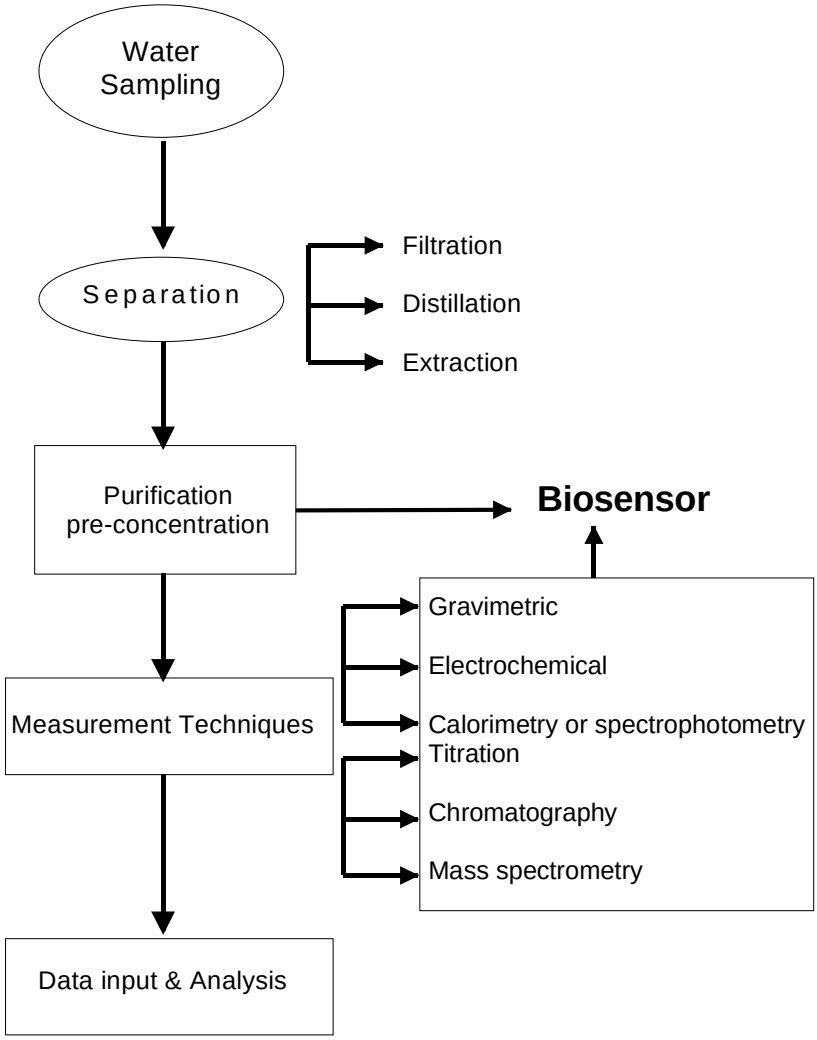


Figure 1.1: Analytical procedure for herbicides with points where biosensor is of use.

1.4.1 Pathway of pesticides in water

Interference of substances emerging from the water matrix is one of the potential problems associated with pesticide analysis from real samples. Pesticides used in agriculture and pesticides used in urban areas (industrial weed control, material protection, etc.) have different pathways to the water reservoirs (Gerecke *et al*, 2002).

Improper use of pesticides could lead to contamination of rivers, lakes and ground waters. Handling and disposing of packing material, filling of sprayers, washing of

utilities, cleaning of spraying equipment are all critical operations that could lead to pollution. The pathway of the resultant pesticides is through sewerage, septic tanks and into surface waters where currents are strong enough to transport them. The pesticides which are stored in sealed surfaces such as farm yards could also be transported through the above mentioned mediums with the first rainfalls. Since the use of pesticides is not always sufficiently controlled, many are not eliminated in waste water treatment plants (Hill *et al*, 1986, Nitschke *et al*, 1999) ending up into the receiving water.

In urban areas, contamination of surface waters may also happened from the same sources. The use of pesticides by inexperienced personnel which includes gardeners, applications on lawns, streets, or road embankments, roads and railways or by home owners, could lead to pollution if the work is not carefully or properly done. Depending on the sewerage systems (households and rain water discharges) pesticides could pass directly into surface waters avoiding any waste water treatment. Since pesticides are characterised by a long lifetime their contamination of drinking water, surface and ground water is almost certain (Tahmasseb *et al*, 2002).

Several techniques exist in order to eliminate pesticides such as ozonation (Legube *et al*, 1987, Benitez *et al*, 1995) and adsorption on activated carbon (coke) (Richard *et al*, 1991, Prados *et al*, 1992) but only a few of these applications such as photochemical degradation are implemented (Héquet *et al*, 2001).

1.4.2 Concentration range of pesticides in drinking water

From the entire water medium, the freshwater one has being the most important as it is an essential and exposed resource of human life. From the global freshwater surface supplies, 70% is allocated and used for agricultural purposes, the largest single use so far. Surface waters which include both rivers and lakes are mainly contaminated by herbicides. During spring and early stages of summer where herbicides have been applied to crops, the herbicides in varying concentrations are found to be higher in surface waters rather than in ground waters. This indicates a seasonal peak which results from their use in fields and the successive runoff that follows. This pesticide runoff is the main source of contamination and usually occurs when rainfalls supersedes soil adsorption. The National Institute of Public Health and Environmental Protection in the Netherlands have

summarised that 65% of agricultural land will exceed the sum (0.5 μg/l) of total pesticides allowed by the EC standard in all countries. An example of pesticides exceeding the maximum level allowed for drinking water has occurred in the UK in the period of 1985 to 1987. At this time the Maximum Admission Concentration (MAC) was exceeded for single pesticides in 298 water supplies and in 70 supplies for the total pesticide level. In 1995, most of the drinking water data at European scale became available and it was founded that approximately 30% of the supplies exceeded the standards set by the EC Directive. Pesticide pollution for most of the member countries is summarised in Table 1.1 and shows a great variability.

Table 1.1: EU data of drinking water pollution in 1995 (Heinz, 1998).

Country	**Drinking water production million m^3**	**% of groundwater resource**	**% Pesticide level > 0.1 μg/l**	**% Pesticide level > 0.1 μg/l in groundwater**
Germany	6052	64	15	15
Austria	450	49	7	15
UK	7620	28	26	15
Italy	8465	48	31	50
France	6080	62	48	40
Denmark	348	99	5	5
Netherlands	1227	69	48	25
Greece	950	68	12	-

In England and Wales, reports published by the Drinking Water Inspectorate (DWI) have shown that in 23 of the supplies the level of herbicides have exceeded the allowed limit in 1996, 15 in 1997, and 12 in 1998 (DWI, 1997). The maximum detected level for the herbicides in 1997 was found to be 0.21 μg/l for atrazine, 0.2 μg/l for diuron, 0.13 μg/l for isoproturon, and 0.14 μg/l for simazine. Table 1.2 indicates in more detail the herbicides that have exceeded the value of 0.1 μg/l in surface waters in the year 1997 for England and Wales.

Table 1.2: Herbicides exceeding water drinking level.

Herbicide	Total number of samples	% of samples > 0.1 μg/l
Isoproturon	3571	17.4
Mecoprop	3526	12.6
Diuron	3759	11.9
MCPA	2120	5.7
Simazine	6284	5.3
Atrazine	6409	4.6
Bentazone	1638	1.5
Chlorotoluron	3619	1.4

1.5 TRIAZINES

1.5.1 Chemical structure and characterisation

Triazine compounds were discovered in the 1950's and constitutes one of the major classes of herbicides. Their structure comprises a single ring with three nitrogen atoms and three carbon atoms (Figure 1.2). They can take both symmetrical and asymmetrical configuration by shifting the nitrogen atoms. Toxicity levels are the same in all structural conformations.

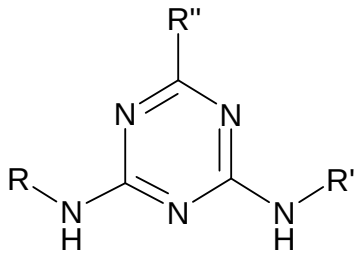


Figure 1.2: Triazine compound structure.

Triazines and especially compounds such as atrazine and simazine are used for control of annual broad-leaved weeds and graize in maize, sugar cane, pineapples, sorghum, conifers and industrial weed (Tomlin, 2003). In U.S., triazines are considered to be the most utilised pesticide compounds. In general, triazine herbicides are the ones with

the higher effect on chloroplasts especially from higher plants. These are the type of compounds inhibiting photosynthesis by targeting the D1 reaction centre of the photosystem-II protein that exists in the thylakoids (Trebst and Draber, 1979).

Among the most common triazines herbicides are atrazine, simazine and cyanazine, with the atrazine being used most frequently in the past. Atrazine is a white crystalline solid with a molecular weight of 216 Da, and solubility in solvents such as chloroform, diethyl ether, dimethyl sulfoxide as well as solubility in water of 28 mg/L at 20^{o}C (Kidd and James, 1991). Atrazine has been found to be moderately toxic in humans and animals. It is readily absorbed through the gastrointestinal tract. Tests on rats have indicated 80% absorption of the atrazine dose to the gastrointestinal tract and later on it is transported to the bloodstream. Atrazine has been used in order to control broadleaf and grassy weeds in corn, sorghum, sugarcane, and pineapple. Furthermore, it has being used in plantings of conifer, Christmas trees and other crops. Atrazine is a herbicide of class-III toxicity. After a special review from the Environmental Protection agency, the use of the compound has been restricted or completely banned. Products containing atrazine should bear the word caution on them.

1.5.2 Environmental fate and toxicity

Environmental fate in general describes the behaviour of the pesticides when it reaches a certain medium. This could be affected by the natural affinity of the chemicals and it influences environmental parameters including solid matter (mineral matter and particulate organic carbon), liquid (solubility in surface and ground water as well as in soil), gaseous form (volatilisation) and living organisms (biota). The factors that need to be considered for herbicide “partitioning” in the environment involve the determination of the soil sorption coefficient (K_{OC}), solubility, Henry’s Law Constant (H), and the n-octanol/water partition coefficient (K_{OW}).

The ecological impact of herbicides in water and the surrounding environment is affected by four main factors. These are toxicity, persistence, fate and degradation of the parent compound. Toxic response (effect) can be acute (death) or chronic (an effect that does not cause death over the test period but which causes observable effects in the test organism such as cancers and tumours, reproductive failure, growth inhibition, teratogenic

effects, etc.). Levels of toxicity for herbicides are measured according to the "lethal dose" (LD_{50}) and the risk factor for their assessment is calculated as the amount of exposure over a length of time times the toxicity.

Triazines in general have low acute toxicity to mammals. The LD_{50} values for atrazine and simazine are in the area of 3 g/kg/day and it is low enough for others compounds in the group. Acute poisoning from triazine compounds includes skin and eye irritation, vomiting and nausea, diarrhea, muscle weakness and salivation. No cases have been reported for acute poisoning from atrazine, simazine, propazine and cyanazine. In case studies of chronic toxicity from oral rat studies, no effects have been found neither visible nor microscopic signs. The fate of triazines in humans and animals is through their gastrointestinal track. Studies on animals have indicated excretion of the chemicals through urine and faeces and only small residues have been bioaccumulated in the fat and muscle of the animals.

1.6 UREAS

1.6.1 Chemical structure and characterisation

Urea compounds are used extensively as herbicides. Their effect depends on the substitution atom (Figure 1.3) and it is the arrangement of this structure that affects the identity and activity of the urea substituted compound. The two major groups of urea substituted compounds used as herbicides are phenylurea and sulfonylurea. The other major one used for insect control is benzoylurea (Kamrin, 1997).

The main representative of phenylureas is diuron. Diuron is a colourless crystaline compound in its pure form with a molecular weight of 233.10 D, and solubility in solvents such as acetone, benzene, butyl stearate as well as solubility in water of 42 mg/l (Kidd and James, 1991). It is slightly toxic to birds with LC_{50} values reported from 1730 to 5000 ppm. Furthermore, diuron is moderately too highly toxic to fish and aquatic invertebrates. In tests of animals, it is indicated that it is excreted in urine and faeces. The breakdown of the compound is quite similar in animals, plants and soil. Low levels have been bioaccumulated in fat, muscle, liver and kidney of tested cattle that were fed with small amounts.

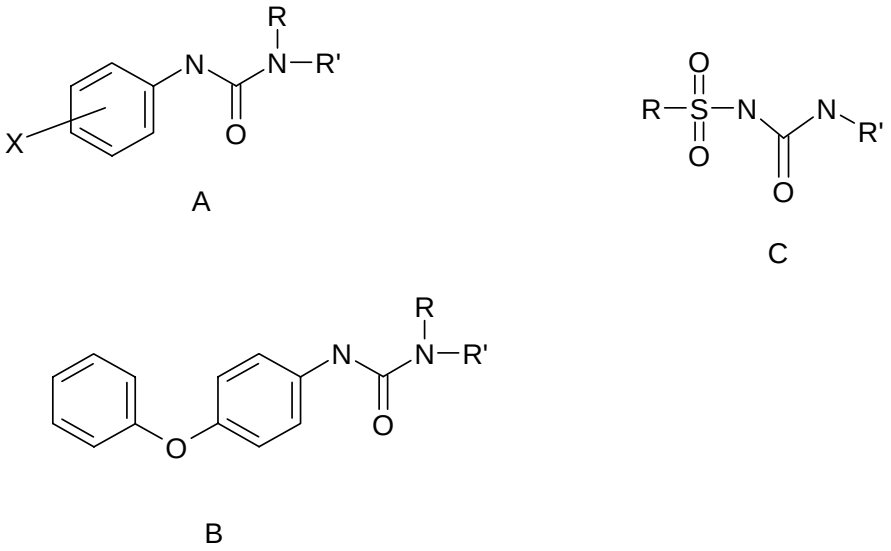


Figure 1.3: Urea chemical structure. A: Phenylurea, B: Benzoylurea, and C: Sulfonylurea.

A case study in pineapple fields has indicated persistence of 3 years after the application of the compound. Metabolites of diuron have more limited mobility than the parent compound and it is related to the type of organic matter in soil and the type of the residue. In an aquatic environment degradation occurs primarily through microbes. However it is quite stable in neutral waters and it is readily absorbed in the root systems of plants and less readily through leaves and stems.

Diuron is considered to be a general use herbicide. The Environmental Protection Agency has classified diuron as a class-III herbicide having slight toxicity. However, a warning has been placed on products containing diuron as it could irritate the eyes and throat. Diuron is used on non-crop areas and on crops such as fruit, cotton, sugarcane, alfalfa and wheat. Furthermore, it is used for a variety of annual and perennial broadleaf and grassy weeds as well as mosses. Diuron has also an effect on chloroplasts from higher plants and therefore inhibits photosynthesis extensively.

1.6.2 Environmental fate and toxicity

Phenylureas exhibit a moderate acute oral toxicity. Generally there are more toxic via the dermal route with LD_{50} levels reaching the 5000 mg/kg in rabbits. The same applies for inhalation tests to rats where toxicity could reach a value of 2 mg/L. Tests have also indicated that symptoms could vary for each phenylurea compounds. Fluometuron and diuron for example have been reported having different effects in rats but it is unknown if this is due to the variation of reporting the symptoms or due to different acts of the compounds on the organisms (Wagner, 1981).

Chronic toxicity of phenylureas includes skin sensitisation to repeated or prolonged exposure. Diuron is the herbicide which has been reported to have teratogenic effects. Tests on rats have indicated skeletal irregularities on exposure to diuron for doses over 125 mg/kg/day for 6 to 15 days period. Overall most class members posses potential for bioaccumulation in animals and humans. Those which are readily absorbed are excreted through faeces. All those that break down inside the body, undergo rapid transformation and elimination through urine (U.S. Environmental Protection Agency, 1983).

All phenylureas compounds remain in soil for the period from few months up to one year and their breakdown is expected to occur due to action of microorganisms in the soil. They usually are not adsorbed by soil having K_{OC} values from 80 to 500 and these results from their tendency of their easy migration in soil. Most of the members of the class of phenylureas have been reported to have half-lives of 25 to over 360 days (Howard, 1991). Their lifetime in water is quite long as well, and they could hardly change hydrolysis, only in extreme acidic or basic environments. In plants, they are absorbed through the roots. They are easily metabolised in tolerant plants forming compounds that could not harm the plants. This occurs through the loss of methyl groups (N-demethylation) and the substitution from the hydroxyl groups (hydroxylation). Plants that could not detoxify herbicides would suffer from their adverse effects.

1.7 PHOTOSYNTHESIS: A BRIEF HISTORY

Photosynthesis is the light energy reaction essential directly or indirectly for all living organisms. It is among the abiotic factors together with temperature, nutrients, and soil structure, which affect growth in higher plants (Lichtenhaler, 1996). Photosynthesis involves oxidation/reduction reactions exactly as do all the energy-yielding oxidation reactions in life (Salisbury and Ross, 1992). The overall reaction includes the release of an oxygen molecule during water oxidation and the formation of carbohydrates during the reduction of carbon dioxide (CO_2). The reverse process is known as respiration and it is an energetically downhill reaction where a strong electron acceptor (O_2) combines with H^+ to form H_2O. In this case, photosynthesis is an uphill reaction to a weaker electron acceptor (CO_2). The light harvesting apparatus that could achieve this uphill transport of electrons is the thylakoid membrane of chloroplasts.

The first photosynthetic reaction was observed in 1864 due to the growth of starch in illuminated chloroplasts. Therefore the overall photosynthetic reaction has been demonstrated as follows:

$$CO_2 + 2H_2O + light \rightarrow CH_2O + O_2 \qquad (1.1)$$

The $(CH_2O)_n$ represents the starch or any other form of carbohydrates. Starch is considered to be the most abundant photosynthetic product of chloroplasts. In 1930s the similarities between plant and bacteria photosynthesis has been noted. Bacteria reduce CO_2 again using light energy but instead of water they could use any other electron source such as acetic or succinic acid. Therefore, photosynthesis in bacteria has the following form:

$$nCO_2 + 2nH_2S + light \rightarrow (CH_2O)_n + nH_2O + 2nS \qquad (1.2)$$

From reactions 1.1 and 1.2 the similarities and the analogy between H_2O and H_2S and the resulting O_2 and S, suggested that the molecular oxygen released during plant photosynthesis results from water and not from CO_2. Once more, in 1930s, Robin Hill's work has demonstrated that isolated chloroplasts or chloroplasts fragments could release

O_2 under light conditions provided with an "artificial" electron acceptor to receive all electrons released from water. This light-driven reaction of water also known as photolysis, has been known as the Hill reaction (Salisbury and Sole, 1992).

1.7.1 Chloroplast structure

Chloroplasts may vary in shape and size according to the variety of plants they belong to (Kirk and Tilney-Bassett, 1978, Wellburn, 1987). Chloroplasts exist in all plants and in certain algae apart from colourless, parasitic angiosperms. Cyanobacteria and other bacterial classes do not have chloroplasts but they do have photosynthetic pigments in their membranes (Salisbury and Sole, 1992). Chloroplasts comprise of a double outer membrane inside which photosynthesis occurs. These double enclosed organelles include additional membranes known as thylakoids. Inside the inner membrane of chloroplasts, there is a mixture of water and enzymes. This is the so called stroma, which comprises embedded stacked sacs known as grana when each sac is known as thylakoid. Each thylakoid has a series of proteins with the most important ones being the photosystems and the light-harvesting proteins that drive the ATP and NADPH synthesis (Chen and Schnell, 1999).

In the past few decades, electron microscopy had revealed the ultrastructural features of membranes from higher plants. Chloroplasts of higher plants have a diameter of approximately 5 μm and a width of approximately 2.5 μm. Each mature leaf cell could contain a few hundred chloroplasts; each of them could contain 40 to 60 granum stacks with diameter of 0.3 to 0.6 μm. These stack regions account for the 50% to 60% of higher plant membranes that grow under light conditions (Staehelin, 2003).

1.7.2 Photosynthetic proteins signal transduction

Signal transduction in bio-macromolecules is the fundamental of life. The conversion of solar energy inside photosynthetic systems involves electron transfer reactions between donors and acceptors which are either immobilised on membranes or free in aqueous phase. This redox character of the reaction in living systems has raised the need to use electrochemical methods for biological apparatus. The z-scheme of photosynthesis indicates in detail the redox potential of the reactions that take place inside

the chloroplasts (Figure 1.4). The recent developments in electrochemical measurements in complex biological systems helped to gain knowledge about this process. Modification of electrodes in order to avoid any interference from metal substances and modification of cells has made electrochemistry a sensitive tool for the study of photosynthesis.

Photosynthetic proteins have been used as current generators in electrochemical cells. This light oxygenic photosynthetic reaction is a result of photophysical, photochemical, and electrochemical events. The photophysical step involves electronic excitation coming from charge separation, while the second step deals with the reactions of excited molecules. Finally, electrochemistry would measure the charge transfer at the interface of the electrolyte solution and the equivalent electrode. Proteins could be directly studied on the electrode surface. There are generally two approaches in the photosynthetic transduction systems. One would involve direct electrochemical measurements by having photochemical proteins directly adsorbed on the electrode surface. The other approach would involve samples to be in suspension and involves the aid of an external electron donor or acceptor. Both approaches were tested in this work using amperometry and cyclic voltammetry as the sensirg technique.

By absorbing light chloroplast thylakoid membranes induce an electron transfer between photosystem I and photosystem II that involves the formation of new redox species (Forti, 1987). These species were detected through a technique primarily designed by Allen and Crane (1976) involving a three electrode photoelectrochemical cell, and later on modified by Carpentier *et al*. (1988). The technique used for the relevant measurements was cyclic voltammetry, which allows the detection of transient species through a wide scan rate. Therefore, this technique provides the relevant information for the stepwise reactions in complex processes such as photosynthesis and respiration. According to previous literature such as the work of Saveant and Viannello (1967) and Nicholson and Shain (1964), in the absence of any artificial electron acceptor and donor, photocurrent is generated by the shared function of photosystem I and photosystem II. Otherwise, PSI would need an artificial electron donor and PSII would need an artificial electron acceptor, in order to work separately.

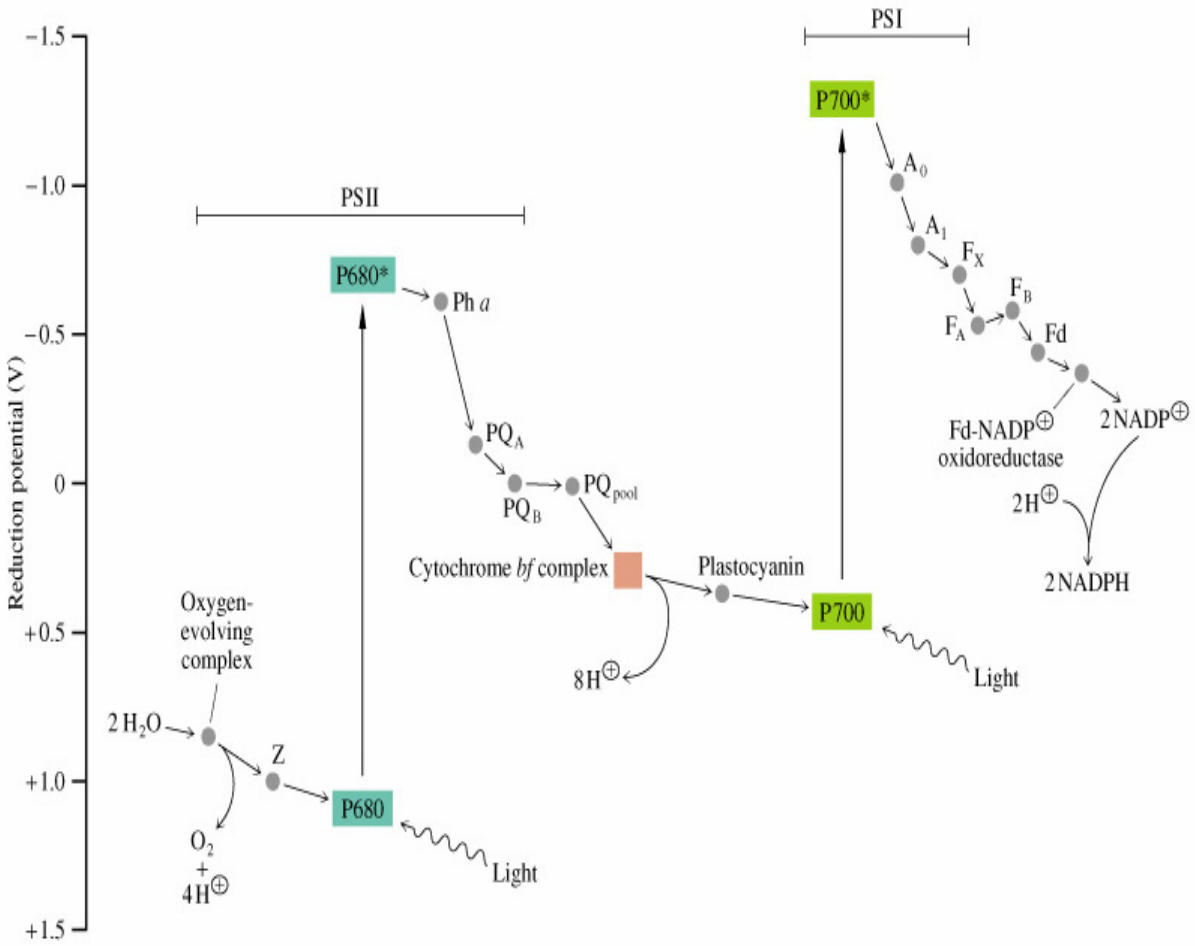


Figure 1.4: Redox reactions in photosynthesis (z-scheme).

CHAPTER 2. BIOSENSOR TECHNOLOGY

2.1 BIOSENSORS

Biosensors can be defined as devices that intimately associate a biological/biomimetic sensing element with a transducer (Bannister *et al*., 2001). These analytical instruments with exclusive capacities combine a recognition power, which naturally exists, in biological systems with sensitivity, flexibility and user-friendliness of advanced microelectronic transducer devices. The role of the latter in a biosensor is to convert an observed change, either physical or chemical into a measurable signal. The magnitude of this signal (usually electrical) is proportional to the concentration of a specific chemical or a set of chemicals. The first biosensor was the one that combined an electrochemical transducer (Clark amperometric oxygen electrode) with enzyme (glucose oxidase) as the sensing element for glucose detection (Cass *et al*., 1984). The Clark electrode is a polarographic electrode used for measuring the concentration of oxygen in blood and gases. The sample is brought into contact with a membrane (usually polypropylene or PTFE - Teflon) through which oxygen diffuses into a measurement chamber containing potassium chloride solution. In the chamber there are two electrodes: one is a reference silver/silver chloride electrode and another is a platinum electrode coated with glass to expose only a tiny area of platinum (e.g. 20 μm diameters). The electric current flow between the two electrodes when polarized with a voltage of 600-800 mV determines the oxygen concentration in the solution (Bannister *et al*., 2001). The principal structure of a biosensor is demonstrated in Figure 2.1.

Among the biological recognition elements enzymes are by far the most important. The reason for this lies in the fact that these molecules provide not only the recognition of analyte-substrate, but also have the catalytic function important for the amplification of the signal (Manahan, 1994). Enzymes are quite flexible molecules and have various complex conformations with sometimes different catalytic activity (Gorton *et al*., 1991). The biorecognition molecules can be integrated in biosensors with a variety of electrochemical transducers (Table 2.1).

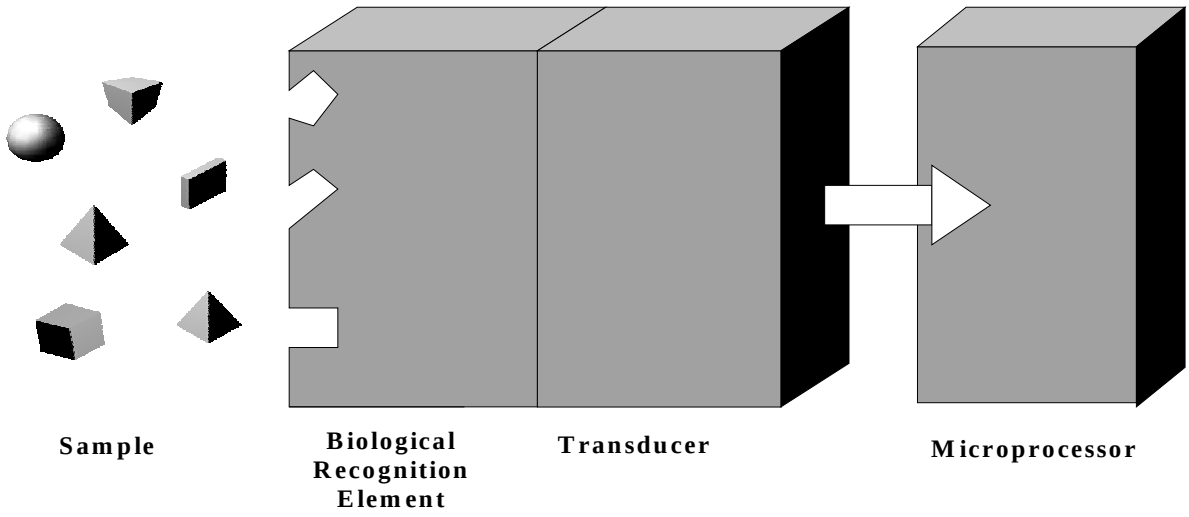


Figure 2.1: A general scheme of a biosensor.

Table 2.1: Comparison of different electrochemical transducers used in biosensors (from Cunningham *et al*., 1998).

Type of energy transduction	Advantages	Disadvantages
Potentiometric	ISE translation is relatively easy Easily miniaturised	Requires reference electrode Limited linear range Often pH sensitive
Amperometric	Wide variety of biochemical redox mechanisms as basis for signal generation Easily miniaturised Good dynamic range, controllable by membrane thickness Relatively good sensitivity	Requires a reference electrode Multiple membranes or enzymes may be necessary for required selectivity and sensitivity
Conductimetric	Easy to fabricate No reference electrode required Low frequency/amplitude source	Non-selective unless used in array format

Among the most popular biosensor devices are amperometric sensors, which usually monitor the change in current at a fixed voltage induced by redox reaction

(Cunningham, 1998). The popularity of amperometric sensors is explained largely due to their simplicity, ease of production and the low cost. A model of an amperometric sensor with chemical transduction by enzymatic reaction is showed in Figure 2.2. The signal in amperometric devices depends on the rate of mass transfer to the electrode surface. In order to minimize the diffusion path of the detectable product of the reaction the enzyme should have close contact with the transducer (Bardeletti *et al*., 1991). Although in some cases it might be possible to monitor a direct electron transfer between enzyme and electrode (Bardeletti *et al*., 1991), normally redox centres of enzymes are located deep in the insulated protein shells, which makes a direct electron transfer unfeasible. The application of such enzymes in biosensors will require the presence of redox mediators. The role and the type of mediators vary according to their usage.

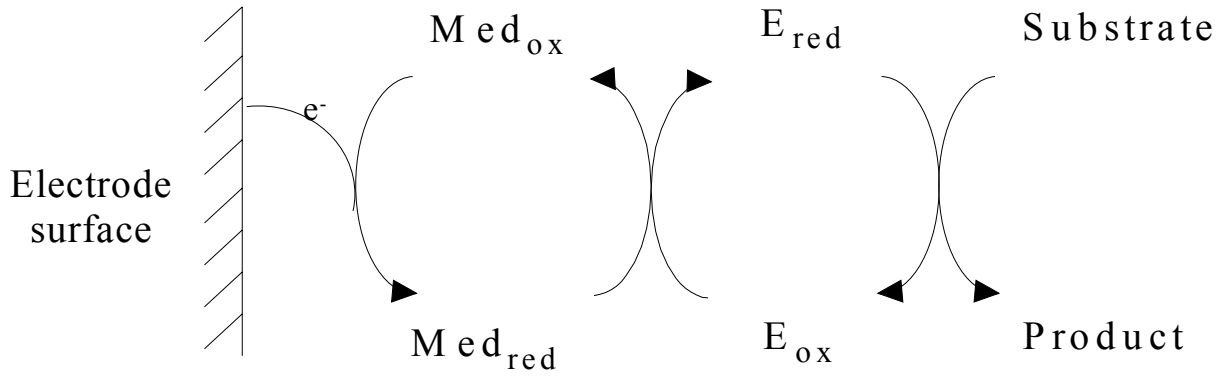


Figure 2.2: Mediated electron transfer, where Med_{ox} and Med_{red} are the oxidised and reduced forms of the mediator, and E_{red} and E_{ox} the reduced and oxidised forms of the enzyme.

2.2 HYDROGEN PEROXIDE - SUBSTRATE AND ANALYTE

Hydrogen peroxide (H_2O_2) is one of the most important products or substrate of enzyme catalysed oxidation reactions (Tang *et al*., 2003; Davis G., 1985). Most common enzymes used in biosensors are oxidases, which catalyse the model oxidation reactions:

$$\text{Substrate S} + O_2 \xrightarrow{oxidase} \text{Product P} + H_2O_2 \qquad \textbf{(2.1)}$$

The function of enzyme is to selectively oxidise analyte by the reduction of O_2 to H_2O_2 (Vreeke *et al*., 1992). Oxygen is the natural electron acceptor that oxidizes use in order to regenerate the enzyme during the reaction. Out of variety of enzymatic reactions that produce H_2O_2 (see Table 2.2), perhaps the most important in practical terms is the oxidation of glucose catalysed by glucose oxidase (GOx). This well-studied reaction, which proceeds according to Equation 2.2 and results in production of H_2O_2, is used extensively in the development of glucose biosensors and assays:

$$\text{Glucose} + H_2O + O_2 \rightarrow \text{Gluconic acid} + H_2O_2 \qquad \textbf{(2.2)}$$

The production of hydrogen peroxide is detected electrochemically and is then related to the concentration of glucose.

In addition to being a product/substrate of enzymatic reaction, hydrogen peroxide is by itself an important analyte. It plays an important role in natural oxidation processes as it is found in air, solids and water. Under different conditions very low concentrations of hydrogen peroxide could be determined, i.e. nano-molar range, in marine waters (Zhang and Wong, 1999), air (Price *et al*., 1998), and drinking water (Lin *et al*., 1998). Furthermore, hydrogen peroxide could be determined at the level of a single cell during oxidative stress in food samples and is used as a substrate in many immunoassays (Jianzhong *et al*.,1994).

The important areas of H_2O_2 application include industry (pharmaceutical, food, clinical), and environmental analyses. Furthermore, its use as an antibacterial agent added to milk, demanding an established protocol for H_2O_2 detection in the food industry. So far the techniques that have been used for the detection of hydrogen peroxide are enzymatic, spectrophotometric, thermo-optic and chemiluminescent assays (Akgol and Dinckaya, 1999). Hydrogen peroxide detection is also possible directly. Most of these techniques are time-consuming and/or suffer from various interferences. Due to this the development of fast, reliable and inexpensive biosensors for hydrogen peroxide detection are of prime importance.

Table 2.2: List of enzymatic reactions, which produce or consume hydrogen peroxide

Enzyme	**Production/consumption of H_2O_2**	**Reference**
Glucose oxidase	Production	Schumb *et al*. (1955)
Uricase	Production	Schumb *et al*. (1955)
Zinc oxide	Production	Schumb *et al*. (1955)
Horseradish peroxidase	Consumption	Vijayakumar *et al*. (1996)
Tyrosinase (polyphenoloxidase)	Production	Petit *et al*. (1995)
Glycolate oxidase	Production	Hale *et al*. (1990)
Sarcosine oxidase or bovine albumin	Consumption	Erlenkotter *et al*. (2000)
Lactate dehydrogenase and lactate oxidase	Indirect production	Young *et al*. (2001)
L-amino acid oxidase	Production	Setford *et al*. (2002)
Catalase	Consumption	Varma and Mitra (2002)
NADPH oxidase	Consumption	Zhou *et al*. (1997)

The goal of the present review is an analysis of the electrochemical sensor approaches used for the detection of hydrogen peroxide. Due to similarity of the subjects the present review discusses both, the development of the biosensors where hydrogen peroxide is a product/substrate of enzymatic reaction and also the development of chemical sensors/biosensors specifically designed for the detection of this analyte. Several technical aspects of the development of sensors for hydrogen peroxide are reviewed in the following chapters: the choice of physical transducer, the choice of enzyme and immobilisation method, and the performance of sensors with respect to response time, sensitivity, linear range, detection limit and operational stability.

2.3 ELECTROCHEMICAL DETECTION OF HYDROGEN PEROXIDE

The concentration of hydrogen peroxide can be measured directly using amperometric detection. A change in H_2O_2 concentration in the medium appears as a variation in the output current. The quantified parameters are magnitude of the sensor

response, response time, and current response. It is desirable to measure signals in conditions when the linear relationship exists between the current value and the analyte concentration. At that point, the reactions are considered to be in steady state when "pseudoequilibrium" occurs between the species close to the sensor and their consumption at the indicative electrode. One of the serious problems associated with measurement of complex analytes is the possible interference of the redox species present in the sample. Several methods have been reported which aimed at reducing level of interference. These methods include use of perm-selective coatings (Sasso *et al*., 1990; Zhang *et al*., 1994), use of artificial mediators (Cass *et al*., 1984), or selective electrocatalysis (Johnson *et al*., 1995; Newman *et al*., 1995).The use of mediators or selective electrocatalysis helps to lower the detection potential to the level when the majority of interfering species are electroinactive (Arjsiriwat *et al*., 2000).

Erlenkotter *et al*. (2000) have used platinum as a working electrode for direct detection of hydrogen peroxide. The reaction that occurs is described below (Equations 2.3 & 2.4).

$$Me(OH)_2 + H_2O_2 \rightarrow Me + 2H_2O + O_2 \quad \textbf{(2.3)}$$

$$Me + 2H_2O_2 \rightarrow Me(OH)_2 + 2e^- + 2H^+ \quad \textbf{(2.4)}$$

Hydrogen peroxide reduces the platinum oxides to metal and the re-oxidation of the metal is performed electrochemically. Detection of hydrogen peroxide in this case depends on temperature, pH and the oxidation status of the platinum electrodes. The research has proved that the oxidation of H_2O_2 requires a stable oxidised surface for the reproducibility of the detection (Erlenkotter *et al*., 2000). The difficulty with the application of these electrodes lies in relatively high price required for their manufacturing and in the high potential required for oxidation. The potential required to dismute (simultaneous oxidation and reduction) hydrogen peroxide on electrode is + 600 - +1200 mV versus a saturated calomel electrode (SCE) (Hendry *et al*., 1990). The potential depends greatly on the nature of the working electrode (platinum, gold, graphite, graphite-polymer composite, etc.). On platinum the oxidation potential is +400 mV (Benneto *et al*., 1987), which is quite high and needs to be reduced in order to avoid any interference coming from real samples (Morales *et al*., 1996). On carbon electrodes, which are much cheaper

than platinum electrodes, the oxidation potential for hydrogen peroxide is even higher (>700mV) (Morales *et al*., 1996). The good compromise on price and performance was achieved for carbon (graphite) electrodes modified with rhodium (Janata *et al*., 1994), mercury or platinum (Cardosi and Turner, 1991). The mechanism of action of these additives lies in the ability of metals such as platinum and rhodium to increase the rate of electron transfer between the enzymes and the conducting sites of the electrodes and speed up the response time of the measurements (Morales *et al*., 1996).

Further reduction of the oxidation potential and enhancement of sensor signal can be achieved by using an enzyme, such as horseradish peroxidase (Schubert *et al*., 1991). The simplest electrode type is the one that consists of a layer of peroxidase molecules adsorbed on the electrode surface (Ruzgas *et al*., 1996). The sensor response, measured at the lowering overpotential of 0.6V vs SCE consists of the change in reduction current which is proportional to peroxide concentration. This type of sensors can be based on carbon black (Yaropolov *et al*., 1979), graphite (Gorton *et al*., 1991; Razumas *et al*., 1984), gold (Yaropolov *et al*., 1979), and platinum electrodes (Diurliat *et al*., 1989; Comtat and Durliat, 1994).

A variety of mediators - small organic molecules capable of lowering the redox potential can be used for facilitating electron transfer between the enzyme catalytic centre and electrode. Mediated amperometric biosensors also have an advantage over non-mediated enzyme electrodes, since the mediator could replace oxygen as an electron acceptor. Hydrogen peroxide detection is known to be affected by oxygen concentration, since the gas is a co-substrate of oxidase-catalyzed reactions (Equation 2.5).

$$H_2O_2 \rightarrow O_2 + 2H + 2e^- \qquad \textbf{(2.5)}$$

In this respect, mediated enzyme electrodes generate more reliable signals, less affected by possible interference from the oxygen in the sample. Furthermore, the employment of mediators with low redox potentials is advantageous in order to operate an enzyme electrode at potentials lower than the ones required for the dismutation (spontaneous oxidation/reduction) of hydrogen peroxide (Hendry *et al*., 1990). This is important for avoiding interference of other electroactive species that could be present in the sample

solution. The details on the development and application of enzyme electrodes in the detection of hydrogen peroxide are discussed in the following chapter.

2.4 ENZYME ELECTRODES, DESIGN AND PERFORMANCE

2.4.1 Choice of enzyme

Using a biological catalyst it is possible to eliminate the problems of interferences by other electroactive compounds. A low operational potential could be achieved by using an enzyme along with a mediator. For peroxidases, the redox enzyme most commonly used for the above situation is horseradish peroxidase. This is the one most likely chosen between great varieties of peroxidases which are able to reduce hydrogen peroxide to water with an associated oxidation of a suitable electron donor. Having into consideration the factors that affect the stability of immobilized enzymes, it is possible to prepare a biosensor that would have longer life. Factors that could cause this type of unsteadiness are organic solvents, and hydrogen-bonding reagents, such as urea, and finally pH and heat (thermal instability) (Johnson *et al*., 1995).

The enzymes that are used intensively in development of hydrogen peroxide sensors are horseradish peroxidase and catalase. Horseradish peroxidase (HRP) along with catalase are both hemic enzymes that contain Fe(III)-protoporphyrin as the prosthetic group. Both enzymes work with hydrogen peroxide as the substrate. Detection schemes vary according to the method of the enzyme immobilisation, mediator, and type of enzyme (see Table 2.2). HRP catalyses the oxidation of numerous substrates (noted as SH) by hydrogen peroxide, following the general reaction:

$$2SH_2 + H_2O_2 \xrightarrow{HRP} 2SH^0 + 2H_2O \qquad \textbf{(2.6)}$$

Direct reduction of hydrogen peroxide at a peroxidase modified electrode is demonstrated in Figure 2.3.

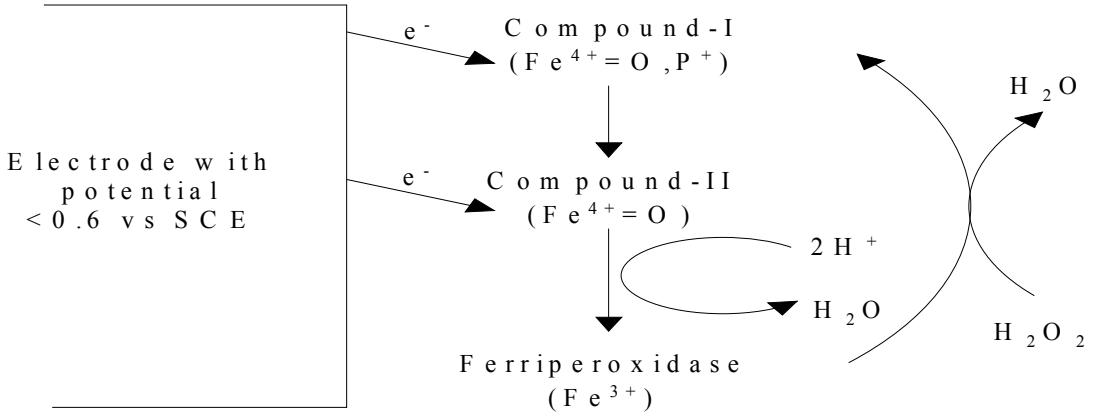


Figure 2.3: Direct bioreduction of hydrogen peroxide using a peroxidase modified enzyme at a potential below than 0.6 vs SCE. Compound-I is the oxyferryl π-cation radical heme intermediate 1 and compound-II is the oxyferryl intermediate 2, where both of them are the oxidised forms of the native ferriperoxidase. P^+ is the cation radical, which is localised on porphyrin ring or polypeptide chain (Nakajima and Yamazaki, 1980).

Reduction of HRP involves compounds I and II, E_1 and E_2 respectively as has been previously described, but its inactivation with excess of H_2O_2 involves the formation of compound-III or E_3 (oxyperoxidase) or the irreversible set of reactions which ends at the formation of a verdohemoprotein (Nakajima and Yamazaki, 1980; Rodriquez-Lopez *et al*., 1997).

Another enzyme which has been applied often is catalase. Catalase is a heme protein belonging in the class of oxidoreductases with ferriprotoporphyrin-IX that acts as the redox centre for the enzyme. As mentioned before, this enzyme catalyses the degradation of hydrogen peroxide by the following reaction:

$$\underset{\text{Catalase}}{H_2O_2 + Fe(III)\text{–}E} \rightarrow H_2O + \underset{\text{(Compound–I)}}{O = Fe(IV) - E} \qquad \textbf{(2.7)}$$

$$H_2O_2 + O = Fe(III)\text{–}E \rightarrow H_2O + O_2 + Fe(III) - E \qquad \textbf{(2.8)}$$

where hydrogen peroxide acts both as an electron acceptor and electron donor (Varma and Mitra, 2001).

2.4.2 Immobilisation

The factors that affect the performance of H_2O_2 biosensor are i) the type of enzyme, ii) the immobilisation method, and iii) the nature and the thickness of the created enzyme layer (Bardeletti, 1991). Immobilisation processes are quite an important factor for the development of biosensors. This step has been studied extensively, in order to achieve easy operation, quick measurement and reduce the cost of the analysis (Bickerstaff G.F., 1997). The methods of enzyme immobilisation used for the development of hydrogen peroxide sensors can be divided into five major groups (Figure 2.4):

- Physical adsorption;
- Covalent binding to an activated insoluble support;
- Encapsulation - capturing biological components with various forms of semi-permeable membranes;
- Entrapment in gels;
- Cross-linking by means of bifunctional reagents (this design could be also used together with adsorption and entrapment).

Adsorption of enzymes has been used extensively for creating chemical transduction layers in a variety of biosensors. Enzymes in general are adsorbed onto metal, metal oxide, carbon and glass surfaces that are used in transducers. The advantages of physical adsorption over other immobilisation techniques are the simplicity of the process along with low cost, little or no damage to enzymes, absence of chemical changes in enzyme structure, which might affect sensitivity and specificity, reversible character, and the possibility of regeneration of the catalytic surface (Woodward, 1985). Sometimes adsorption could be used along with cross-linking (Blum, 1997). This procedure has several steps such as the mixing of the biological and chemical components under optimised conditions of pH, temperature and ionic strength followed by a period of incubation during which the chemical reaction proceeds. Immobilised materials are washed thoroughly for the removal of the non-bound biological components that would easily interfere in the solution and distort the data.

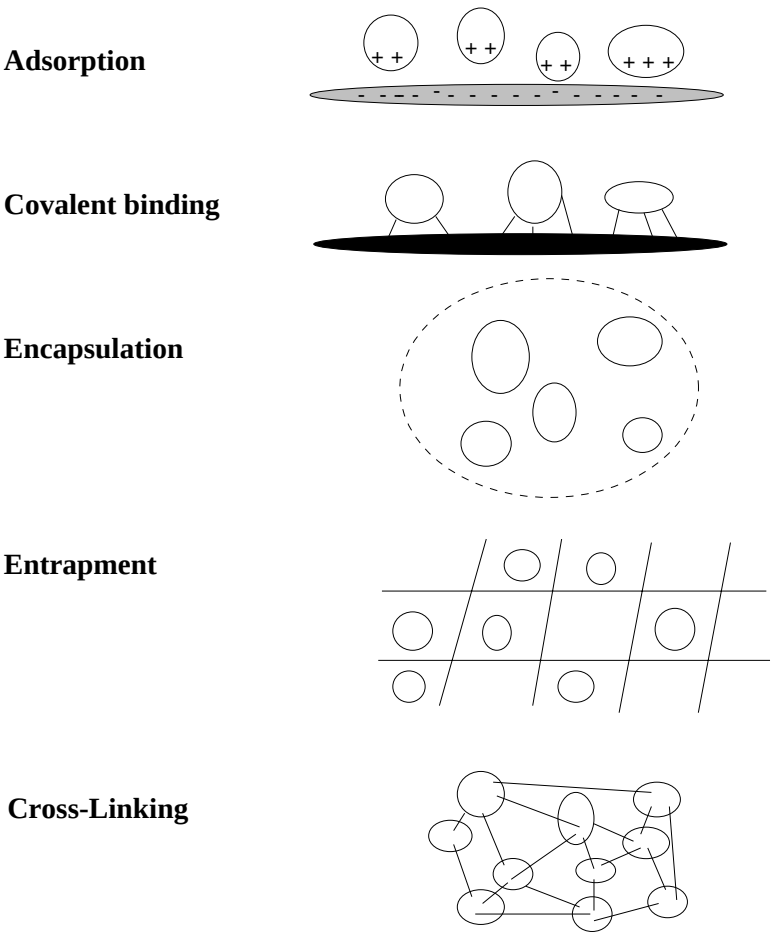


Figure 2.4: Schematic diagram of the principal immobilisation techniques (from Bardeletti *et al*., 1991).

Covalent binding involves the formation of a covalent bond between the functional groups of enzyme and the support material. Proteins are bound through the involvement of amino, carboxyl, sulfhydryl, or aromatic parts of the chains of the amino acid in the macromolecule (Cunningham A., 1998). Some examples of amino acids group suitable for covalent binding are the amino group (NH_2) of lysine or arginine, as well as the carboxyl group (COOH) of aspartic acid or glutamic acid, the hydroxyl group (OH) of serine or threonine, and the sulfydryl group (SH) of cysteine (Bickerstaff G.F., 1997). The popular process for activation of functional groups and covalent enzyme immobilisation is the formation of a Schiff base, catalysed by carbodiimide. The hydrophilic factor of the supporting material is an important factor, which determines the efficiency of immobilisation. Polysaccharide polymers are very hydrophilic and some of them such as cellulose, dextran (Sephadex), starch, agarose, (Sepharose) have been extensively used for enzyme immobilization (Bickerstaff G.F., 1997).

Encapsulation achieves the confinement of biological components by using various semi-permeable membranes (Bilitewski and Turner, 2000). Encapsulation allows the enzymes to exist freely in solution, which is confined within the small area surrounded by the membrane. Macromolecules cannot cross the membrane barrier, which is permeable for small molecules only (substrates or products). Nylon and cellulose nitrate are the most popular materials used for the production of microcapsules that need to have a diameter between 10 and 100 μm. Furthermore, biological cells could be used as capsules as shown in erythrocyte based sensor (Bickerstaff G.F., 1997). Alternatively enzyme solution can be encapsulated in a thin layer, which covers the electrode and confined between the electrode and semi-permeable membrane surface (Cabral and Kennedy, 1991).

Entrapment differs from the above-mentioned techniques due to the fact that the movement of immobilised molecules is restricted by the presence of a gel. The porosity of this gel controls the enzyme mobility and the gel should be tight enough to avoid any leakage of the enzyme. Although the technique sets a barrier to the mass transfer and this would affect the reaction kinetics, the advantage is that the enzyme does not interact with the immobilized biocatalyst (Brodelius, 1985). In some cases such as hydrogels, their swelling property would allow the enzymes to go through and leave the gel which could limit their application (Wang and Dong, 2000). Apart from gels, entrapment could use a variety of organic and inorganic polymers produced on the transducer surface by electrochemical, photochemical, or plasma polymerisation. One such example involves enzyme entrapment into solidified materials such as paraffin. Petit *et al*. (1995) studied the hydrodynamic conditions of paraffin and how it improves the stability of the enzyme-immobilised electrode and the reproducibility and repeatability of the results. Many researchers have used entrapment of the enzyme into silica polymer that contains weak hydrogen bonds hybridised into sol-gels (Pankratov and Lev, 1995; Cunningham, 1998). The fabrication of a biosensor with sol-gel is straight forward and could be done by syringing a mixture of the sol-gel and the enzyme solution on the electrode and allowing water to evaporate (Wang and Dong, 2000). These organic and inorganic materials are able to incorporate both, mediators and enzymes for the fabrication of biosensors (Zhang *et al*, 1999). They could be prepared under ambient conditions and they have good porosity, high thermal and chemical stability and insignificant swelling in aqueous and non-aqueous solutions. The main limitation of sol-gel immobilisation techniques lies in

the relatively low stability of inorganic membranes, which have a tendency to cracking. In order to overcome this problem Wang and Dong. (2000) and Zhang *et al*. (1999) have used hybrid inorganic-organic materials. Polyvinyl alcohol (PVA) and hydrophobic poly(vinylpyridine) (PVP) have been used to compose the sol-gel composite material for developing a hydrogen peroxide biosensor (Wang and Dong, 2000).

The final immobilisation procedure that has been used is cross-linking and involves joining the enzymes to each other forming a large, three-dimensional complex structure, achieved by physical or chemical methods. The chemical methods of cross-linking involve covalent bond formation between the protein molecules by means of a bi-multifunctional reagent such as glutaraldehyde and toluene diisocyanate (Bickerstaff *et al*., 1997; Sarkar *et al*., 1999).

Apart from being a framework, the immobilisation matrix could have additional functions such as selective ion permeability, enhanced electrochemical conductivity, or mediation of electron transfer processes (Cunningham, 1998).

2.4.3 Mediators

Using mediators in combination with enzymes is a practical alternative for the direct measurement of hydrogen peroxide and for the direct monitoring of enzymatic reaction. Another advantage is that the use of mediator could help to overcome the dependence of the amperometric biosensors on dissolved oxygen since the mediator can provide the essential electrons (Vreeke *et al*., 1992).

In general, the mediated signal coming from an enzyme electrode is monitored through the measurement of the quantity of reduced redox mediator. In the case of horseradish peroxidase (HRP) based electrode the catalytic reaction that produces that mediator is described below:

$$H_2O_2 + 2H^+ + HRP_{red} \rightarrow 2H_2O + HRP_{ox} \quad \textbf{(2.9)}$$

$$MED_{ox} \xrightarrow{E} MED_{red} \quad \textbf{(2.10)}$$

The HRP_{red}, HRP_{ox}, MED_{red}, MED_{ox} are the reduced and oxidised forms of the enzyme and the mediator, respectively.

A variety of compounds have been used as mediators in enzyme biosensors (Table 2.3). The most common mediators used for hydrogen peroxide and glucose detection are ferrocene and its derivatives (Cass *et al*., 1984; Vidal *et al*., 1994; Sadeghi *et al*., 1997). In reaction with HRP, H_2O_2 is oxidised to water by enzyme and ferrocene acts as an electron donor to the oxidised iron in the haem protein. The charge that is involved for the reduction of the ferricium ion on an electrode is proportional to the hydrogen peroxide concentration. Tatsuma *et al*. (1989) used soluble ferrocenemonocarboxylic acid as mediator, which operated at a potential of +150 mV vs. Ag/AgCl. Ferrocene derivatives have been highly utilised since they exhibit excellent properties such as maintaining a one-electron redox couple even when their cyclopentadienyl rings are modified with several other substituents. In this way they can be used for modifying protein molecules. An another group of mediators with similar properties are π-cyclopentadiennyl-π-dicarbollyliron complex ($CpFeC_2B_9H_{11}$), and pentaamminepyridineruthenium(II) complex $[Ru(NH_3)_5py](PF_6)]$ used by Frew *et al* (1986).

Zhang *et al*. (1999) have prepared a H_2O_2 sensor using Meldola's Blue (MDB) as mediator. MDB was incorporated into hydrogels in order to give a faster reaction rate. Other mediators used for the detection of hydrogen peroxide are phenothiazine molecules such as methylene blue (Lei and Deng, 1996; Qian *et al*., 1995), methylene green (Liu *et al*., 1996), thionine (Ruan *et al*., 1998; Xu *et al*., 1998), $[Os(bpy)_2pyCl]^+$ (Dequaire *et al*., 2002), and toluidine blue (Rajendran *et al*., 1998). Mediators are able to shuttle electrodes between electrodes and enzymes in several configurations such as soluble, associated in monolayer or multilayer, or incorporated in porous matrices. In all the cases it is necessary for the mediator to be regenerated having the appropriate medium and for the enzyme to play the role of a biocatalyst.

Table 2.3: Classes of mediators that have been used for amperometric detection of hydrogen peroxide detection.

Mediators	Ox/Red potential(mV)	Ref
Anthraquinones	-40	Saby *et al*. (1995)
Methylene Blue	-156	Saby *et al*. (1995)
p-Benzoquinones	+200	Casero *et al*. (2000)
Ferrocene carboxylic acid	+295	Sadeghi *et al*. (1997)
Ferrocene dicarboxylic acid	+420	Sadeghi *et al*. (1997)
Aminoethyl ferrocene	+158	Sadeghi *et al*. (1997)
N,N,N-Trimethyl-N-ferrocenomethylammonium iodide	+137	Sadeghi *et al*. (1997)
1,1'-Bis(hydroxymethyl) ferrocene	+220	Sadeghi *et al*. (1997)
Hydroxymethyl ferrocene	+180	Sadeghi *et al*. (1997)
1,1'-Dimethyl-3-ethanolaminoferrocene	+490	Sadeghi *et al*. (1997)
Hexacyanoferrate (III)	-100	Miao and Tan (2001)
Ru, Os complexes	+500	Reiter *et al*. (2001)
Methylviologen MV	-660	Brunetti *et al*. (2000)
Charge transfer complexes (e.g. tetracyano-p-chinone dimethane TCNQ-tetrathiafulvalene TTF)	+200	Sekine and Hall, (1998)
ABTS2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)	-200	Kadnikova and Kostic, (2002)
Meldola's Blue (MD) within inorganic/organic hybrid material	-116	Zhang *et al*. (1999)
Ferrocenemonocarboxylic acid	+150	Tatsuma *et al*. (1989)
Ferrocenedicarboxylic acid	+150	Tatsuma *et al*. (1989)
Ferrocenecarboxaldehyde	+100	Tatsuma *et al*. (1989)
Pottasium ferrocyanide	+100	Tatsuma *et al*. (1989)

Since the mediators should be mobile in order to provide the electron flow between the enzyme catalytic centre and electrode, they are usually soluble in the electrolytic medium, and therefore could be lost during the repetitive measurements. This, in combination with inactivation of enzyme, due to its denaturing leads to the loss of sensitivity of the biosensor with time. The possible solution to this problem is using an

excess of enzyme and mediators in the measurement (Morales *et al*., 1996). Recently, a new technique has been developed where redox polymers were used in dual function: as immobilisation matrix and as materials facilitating electron-transfer (Garguilo *et al*., 1993). In materials such as these, the mediator redox couple is not diffusing and therefore the electron transfer occurs by "hopping". Such a mediator is not leaching out of the membrane of the sensor and therefore the use of a containment membrane is unnecessary. An example of this redox polymer material has been used by Hale *et al*. (1990) for a glycolate sensor. The polymeric electron transfer mediators that were used were siloxane polymers with covalently attached ferrocene and 1,1-dimethylferrocene. With this procedure, electron transfer was efficiently transmitted from the reduced glycolate oxidase to the carbon-paste electrode. Garguilo *et al*. (1993) have developed amperometric sensors for hydrogen peroxide by immobilizing horseradish peroxidase (HRP) in a cross-linked redox polymer deposited on glassy carbon electrodes. The redox polymer contained Os derivatives such as (PVP-$O_s(bpy)_2Cl$; bpy- 2,2'-bipyridine, PVP-poly(4-vinylpyridine) and $O_s(bpy)_2pyCl$ (PVP replaced with pyridine) covalently attached to the HRP enzyme.

2.4.4 Examples of enzyme biosensors used for the detection of hydrogen peroxide

Tatsuma *et al*. (1989) have immobilized HRP in a monolayer covalently attached to a tin oxide electrode. The sensor used soluble ferrocenemonocarboxylic acid as mediator, which operated at a potential of +150 mV vs. Ag/AgCl. The electrode was chosen for its chemical and electrochemical stability as well as for the simplicity of modifying its surface with functional groups. The sensor had enhanced kinetic rate and as result increased sensitivity, with detection limit 10^{-8} M H_2O_2. The immobilisation of the enzyme as a monolayer could reduce also fabrication cost (which is especially important for the case when the enzyme is expensive).

Frew *et al*. (1983) developed H_2O_2 sensors using π-cyclopentadiennyl-π-dicarbollyliron and $[Ru(NH_3)_5py(PF_6)]$ complexes as mediators. Their biosensors exhibited sensitivity in the range of $5 \times 10^{-8} - 6 \times 10^{-6}$ M to hydrogen peroxide using a gold or pyrolytic graphite electrode.

Zhang *et al*. (1999) have prepared a H_2O_2 sensor using MDB as mediator and electrode functionalised with a horseradish peroxidase-containing membrane. MDB was incorporated into hydrogels in order to give a faster reaction rate. Their biosensor demonstrated a linear range in 1-0.6 mM concentration and good stability. The response time of the sensor was less than 25 seconds with a sensitivity of 75 nA μM cm^{-2}.

Li *et al*. (1996) have prepared an amperometric biosensor for hydrogen peroxide using a sol-gel immobilisation of horseradish peroxidase. HRP was entrapped in a thin silica sol-gel matrix made from tetramethoxysilane polymerised on carbon paste electrodes. The hydrogen peroxide detection was performed in the presence of hexacyanoferrate (II). Under optimised conditions, the linear range for detection of hydrogen peroxide was 2 x 10^{-5} - 2.6 x 10^{-3} M. The enzyme electrode maintained up to 65% of its activity for the period of 35 days.

Wang *et al*. (2000) used HRP entrapped into hybrid PVA/silica sol for the measurement of hydrogen peroxide. Their biosensor demonstrated a linear range in the concentrations 0.2-3.4 mM (curve at 3.4 mM in figure 2.5), with detection limit of 5 x 10^{-7} M. Furthermore, this biosensor exhibited high sensitivity (15 μA mM^{-1}) and fast response time (10 sec.). The results that were calculated from the calibration curve were in close agreement with the ones measured by a standard spectrophotometric method (Table 2.4).

Sergeyeva *et al*. (1999) have also used HRP along with ttb-CuPc (tetra-tert-butyl-copper phtalocyanine) thin films for the detection of H_2O_2. Their biosensor achieved the detection in the range of 5 - 300 mM and an operational stability of 7 hours, which was improved to 90 days by storing the sensor at 4 ^{o}C. To minimise any interference from the aqueous media the ttb-CuPc layer was covered by a hydrophobic gas-permeable membrane. Due to this, the ionic strength and the buffer capacity did not play a significant role on the measurements. Thus, the iodine that resulted from the peroxidase reaction could be monitored with the aid of the designed transducer.

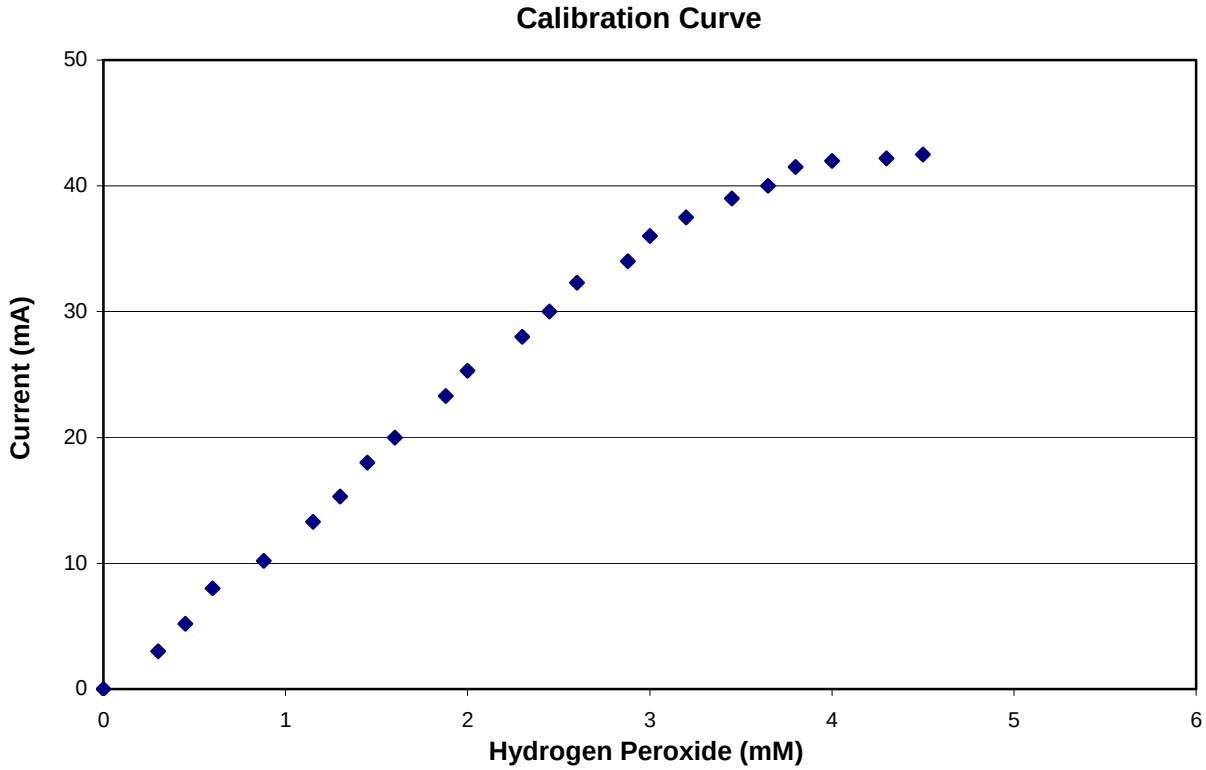


Figure 2.5: Calibration curve for the enzyme electrode for H_2O_2. The potential for the electrode is –50mV and the electrolyte is 50 mM sodium phosphate buffer, pH 7.5 (Wang *et al.*, 2000).

Miao and Tan (2001) have fabricated an amperometric H_2O_2 biosensor using HRP and hexacyanoferrate (II) entrapped into silica sol-gel/chitosan film on the surface of carbon paste electrode (Miao and Tan, 2001). The sensor operated at low potential (-100 mV) and had a similar sensitivity ($2.5 \times 10^{-4} \div 3.4 \times 10^{-3}$ M) as the one prepared by Wang *et al* (2000). Their biosensor exhibited quite good reproducibility and stability. 85% of the original enzyme activity was preserved after 30 days of storage in a phosphate buffer solution at 4^{o} C. The authors claimed that the high stability originated from the amino containing sol-gel/chitosan hybrid films, which provided a hydrophilic environment compatible with the biomolecules.

Wang and Dong (2000) developed an amperometric biosensor using a Nafion-methylene green modified electrode. Nafion is a very popular electrode anion-exchange modifier and has been used in many applications (Gorton *et al.*, 1991). Methylene green (soluble mediator) was incorporated into the Nafion thin layer and it was coated on the

surface sol-gel-enzyme thin film immobilized on a glassy carbon electrode. The electrode revealed a sensitivity of 13.5 μA mM^{-1} with a detection of 1.0 x 10^{-7} M H_2O_2. The sensor response was fast reaching maximum in 20 s. The sensor response was influenced by the pH of the buffer with maximum observed at a pH of 6.5. Sensor had a good long-term stability.

Table 2.4: Comparison of hydrogen peroxide concentrations in real samples between Wang's (2000) biosensor and spectrophotometric method (Frew *et al*, 1983).

Sample No	Concentration of hydrogen peroxide (μM)	
	Measured by biosensor	Determined by spectrophotometric method
Milk		
1	21.16+0.37	21.94+0.29
2	12.05+0.16	12.35+0.13
3	18.31+0.26	18.71+0.21
4	20.09+0.33	20.62+0.27
Acid milk		
1	25.96+0.42	26.33+0.35
2	19.34+0.21	19.56+0.26
3	21.89+0.28	22.16+0.27
Juice		
1	8.46+0.18	8.75+0.12
2	9.39+0.20	9.56+0.11
3	5.22+0.16	5.54+0.08

Yabuki *et al*. (1999) have prepared a H_2O_2 sensor using ferrocene as mediator. Their biosensor was fabricated with a poly-ion complex membrane containing physically entrapped microperoxidase (MP) and ferrocene and immobilized on glassy carbon electrode. The polyion membrane consisted of poly-L-lysine and poly(4-styrene sulfonate). Microperoxidase (heme peptide; heme octapeptide; nonapeptide and undecapeptide) has smaller molar mass than peroxidase (POD) and therefore a higher specific activity. The potential that was applied for this biosensor was 0.0V versus Ag/AgCl and the response current showed linear proportionality up to 20 μM H_2O_2

concentration. The electrode could be used for the period of 10 days and with a low detection limit of 0.5 μM (S/N=5). The calibration curve for hydrogen peroxide is shown in Figure 2.6.

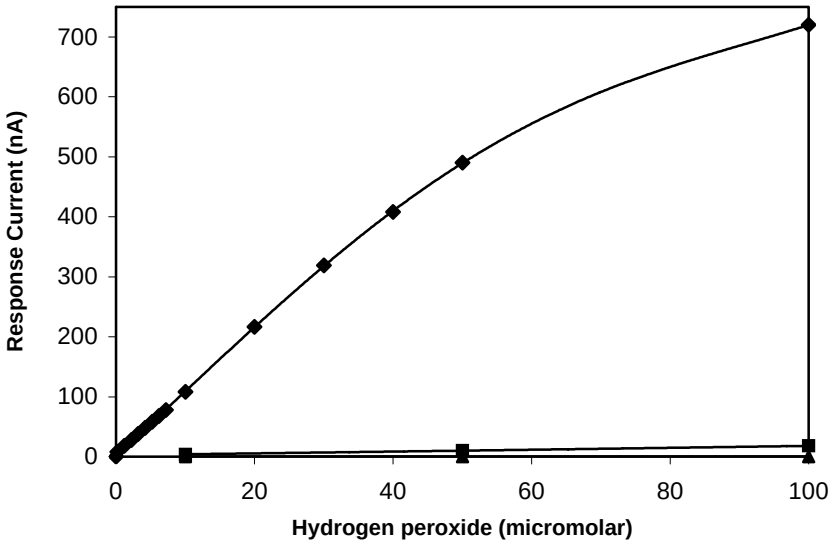


Figure 2.6: Hydrogen peroxide calibration curve for three different electrode systems: MP and ferrocene-immobilized polyion complex membrane (a), ferrocene-immobilized poly-ion complex membrane (b), and bare glassy electrodes (c).

Karyakin and Karyakina (1999) have developed a hydrogen peroxide sensor, based on Prussian Blue deposited on glassy carbon electrodes. Prussian Blue was considered an "artificial peroxidase" due to its high catalytic activity and selectivity, which could be compared with biocatalysis. The application of Prussian Blue modified electrodes enabled the sensing of H_2O_2 at around 0 V vs. SCE. The electrocatalytic reduction of H_2O_2 in the presence of O_2 was found to be better for Prussian Blue deposited on glassy carbon electrodes than for platinum covered electrodes. Furthermore, these electrodes were more stable and active and less expensive than the platinum and peroxidase modified electrodes. The response was linear up to 0.1-100 μM and the detection limit found to be 10^{-7} M.

It is interesting that the enzyme electrodes could be used for the detection of hydrogen peroxide also in organic solutions. Vijayakumar *et al*. (1996) have used carbon paste electrodes modified with HRP, chemically derivatised with mPEG. Covalent

attachment of mPEG to the enzyme helps in preventing enzyme denaturing in organic media, which makes sensor operational in organic solvents such as benzene, toluene and chloroform (Inada *et al*., 1986). In general, measurement in organic phase has advantages such as the possibility to monitor hydrophobic substrates, to remove microbial contamination, to avoid the possible side reactions, and to enhance the thermostability. However, the drawback for these sensors was that the electrode response for H_2O_2, based on direct electron transfer between the electrode and peroxidase is reduced. Additionally, enzymes could not maintain catalytic activity in an absolute non-aqueous environment; therefore, enzymes on organic phase enzyme electrodes (OPEEs) must retain a thin aqueous film in an organic media, which is difficult to control (Dong and Guo, 1994).

The use of polymers in conjunction with metals and enzymes has been proved to be an appropriate alternative for the detection of hydrogen peroxide in both organic and aqueous media. Daly *et al*. (1999) have used three polymers (polypyrrole, polyaniline, and 1,3-diaminobenzene) along with ruthenium (Ru), rhodium (Rh) and palladium (Pt) in several combinations, to provide a suitable H_2O_2 biosensor. Both conductive (Umana and Waller, 1986; Trojanowicz and Krawczynski vel krawczyk, 1990; Hoa *et al*., 1992; Van Os *et al*., 1996; Foulds and Lowe, 1988) and non-conductive (Madaras *et al*., 1996; Sasso *et al*., 1990; Manowitz *et al*., 1995) polymers could be used in electrochemical sensors but the latter are mostly used for blocking interferons or immobilising the biocomponents since they are not electroactive. Daly *et al*. proved that by the correct choice of potential and polymer, an enhanced electrochemical sensor could be obtained. In conjunction with the appropriate metal, the sensor would keep the signal of the interferons low and would enhance the response of the signal of interest being hydrogen peroxide.

Transition metal phthalocyanines on carbon paste electrodes have been also demonstrated for the determination of hydrogen peroxide using differential pulse cyclic voltammetry (Santamaria *et al*., 1998). This type of compounds has been successfully used for antioxidants, substances that contain sulphur, reduced glutathione, organic peroxides, thiocyanate and selenocyanate. The versatility of metal phthalocyanines explains why Santamaria *et al*. (1998) used them for observing the electrochemical behaviour of hydrogen peroxide. Carbon paste electrodes modified with nickel (II) phthalocyanine and copper (II) phthalocyanine showed a greater sensitivity and response than the non modified carbon paste electrodes, at pH 11.

Another possibility for hydrogen peroxide determination is by electrocatalysis of conducting polymers on the electrode surface. During this technique, three processes take place:

1. Heterogeneous electron transfer between the electrode and the conducting polymer layer and electron transfer within the polymeric film.
2. Diffusion of solution species to the reaction zone, where electrocatalysis occurs.
3. Heterogeneous chemical reaction between the solution species and the conducting polymer.

During the first process there is movement of charged compensating anions and solvent molecules within the polymer and there is possible change in the structure of the polymer. The procedures that could affect the rate of this process are the conductivity of the polymer film, the rate of the electron exchange between the polymeric chains, and the anion movement within the film. If electrocatalysis occurs in the polymer film, then the diffusion of the species within the film and the possible electrochemical interactions of these species with the polymer film should be taken into consideration. Finally, the complexity of these procedures does not allow the simple interpretation of the kinetic behaviour or the voltammetric responses.

Electropolymerisation of polymers directly onto the surface of the electrodes has been used for the development of enzyme-based biosensors. A multifunctional polymer film could be fabricated by depositing the monomer solution containing the enzyme and/or any other component of the sensor. The latter are entrapped on the electrode surface as the polymer film grows on top of them. Regardless of the above mentioned disadvantages, with electropolymerisation it is able to control the film thickness through the amount of polymerisation charge, and the polymer film is only deposited on the sensing electrode surface.

Despite the difficulty of electropolymerisation, Yang and Mu (1997) have immobilised HRP in polyaniline (PANI) films both on platinum and glassy carbon electrodes. The response of these sensors was fairly fast and sensitive to hydrogen peroxide concentration and with an operation potential of either 200mV or 75mV. The response was linear to concentration below 5 μM. Another case was by Iwuoha *et al*.

(1997) who deposited HRP on a platinum disk electrode and developed a hydrogen peroxide sensitive biosensor that could be operated both in aqueous solutions with a potential of -100mV vs. Ag/AgCl and also in organic phases such as acetone, acetonitrile, propanol and tetrahydrofuran.

One can conclude that the use of amperometric and potentiometric biosensors has achieved a considerable progress in the determination of hydrogen peroxide. The redox-enzyme and electrode structure achieved by many provides the basis for electrochemical biosensors. Enzymes need to be modified either by mutagenesis, or site-specific reactions that would provide structures with readily accessible sites. Many of the latter could be accomplished with the aid of a mediator. On the contrary, with the aid of polyaniline and polypyrrole film, detection of hydrogen peroxide is possible even at a mediator free solution and at a low potential of -0.1 to 0 V. In this case, the high operational stability of enzymes and the fast response could make the determination possible even with a low amount of enzyme. Schubert *et al*., (1991), used HRP adsorbed on pyrographite but the sensor was unstable due to desorption of the species. In order to get better stability it is necessary to do electrolysis of buffer solutions containing pyrrole and HRP on platinum and SnO_2 electrode materials.

2.4.5 Comparison between metallised and enzyme biosensors.

In order to make a selection between metallised or enzyme biosensors it is necessary to take into consideration the specific features of a biosensor transducer (Lowe, 1985). This includes 4 issues such as:

- High specificity for the analyte at relevant concentrations.
- Fast response time (usually 1-60sec).
- Suitable design for field application.
- Not adversely affected by environmental changes like temperature.

From the pastes used in electrode fabrication, carbon is the most attractive one. This is due mainly to its wide potential range (depended on method), low electrical resistance, low background current and relatively low cost (Wang *et al*., 1996).

Despite these advantages, its printability and reproducibility could be poor since the required carbon amount should be at a low level to obtain a relatively uniform surface.

Furthermore, its use depending on application should be associated with other material. In the case of hydrogen peroxide, the potential used is relatively high (Wang *et al*., 1996, Hendry *et al*., 1990) but a simple modification of the surface with either chemical or biological components, could enhance the selectivity and sensitivity of the sensor. A chemical modification (one used in this case), could reduce the overpotential by providing a better electron transfer between the electrode and the biological component (usually an enzyme) Erlenkotter *et al*., 2000. In amperometric detection, the optimum potential used should be in the area of -100mV to 0mV since the common interferent compounds found in the environment are neither oxidised nor reduced at this potentials (Akgol and Dinckaya, 1999).

Additionally, a way to reduce the potential for amperometric detection of hydrogen peroxide would be by using noble metals such as gold, platinum, rhodium, or metallised carbon which would cost even less (Kröger & Turner, 1997, Newman *et al*., 1995, Bennetto *et al*., 1987, Morales *et al.*, 1996). What noble metals bound to carbon have, are synergistic and electronic interactions. Metallised carbon electrodes are able to minimise interferents' problems by working at lower potentials and increase sensitivity of sensor without any complicated manufacture (Wang *et al*., 1995).

2.4.6 Examples of PS-II based biosensors with hydrogen peroxide production.

Hydrogen peroxide is known do be the product of the dismutation of O_2^- (free oxygen radical). This is the result of the oxidisable electron acceptors of photosystem I in a reaction that includes ferredoxin and NADP reduction to NADPH. However, several biosensors have being previously reported the production of hydrogen peroxide from photosystem II. The latter involves treatment of chloroplasts with hydroxylamine (NH_2OH) in order to oxidise H_2O_2 to O_2 through photosystem II.

Two examples have been reported involving the production of hydrogen through photosystem II. Both of them are based on the same principle of chloroplasts treatment. Nitrite and hydrogen peroxide are produced from the presence of hydroxylamine and the absence of artificial electron acceptors in the following reaction:

$$NH_2OH + 2O_2^- + H^+ \rightarrow NO_2^- + H_2O_2 + H_2O \quad \textbf{(2.11)}$$

Furthermore, the products of the equation 11 depend strongly on the concentrations of dibromothymoquinone and 2,3-dimethyl, 5,6-methylenedioxy p-benzoquinone used separately (Elstner and Kramer, 1973). In the past, hydrogen peroxide has been reported to be a product of oxygen dismutation ($O_2^{\cdot-}$) through auto oxidation of reduced dyes via photosystem I (Elstner and Kramer, 1973; Epel and Neumann, 1973).

Pan and Izawa have used a Clark-type electrode in order to measure oxygen evolution or consumption through photosystem II. This was based again on treatment of chloroplasts with hydroxylamine that would inactivate the O_2 evolving enzyme of the protein. An electron acceptor has also been used such as 2,5- dimethylquinone in the presence of dibromothymoquinone which is an antagonist of the plastoquinone and therefore it could eliminate the presence of photosystem I (Pan and Izawa, 1979).

2.5 CONCLUSION

Since hydrogen peroxide is the product of reactions catalyzed by a large number of oxidase enzymes and is essential in food, pharmaceutical, and environmental analysis, its detection was and remains a necessity. Many attempts have been made in order to develop a biosensor that would be sensitive, stable, inexpensive and easy to handle. The most popular and efficient of them are amperometric enzyme biosensors, which utilized different types of mediators and enzymes, mostly peroxidase and catalase. Unfortunately many of the developed sensors do not meet the requirements for a practical device which has a balance of technological characteristics (sensitivity, reliability, stability) and commercial (easy of mass production and low price). Thus a window of opportunity still remains open for a future development. We hope that the present work will inspire other researches for further advances in the area of biosensors in particularly sensors for detection of such an important analyte as hydrogen peroxide.

CHAPTER 3. FABRICATION, CHARACTERISATION, AND DEVELOPMENT OF SCREEN PRINTED ELECTRODES

3.1 INTRODUCTION

The development of biosensors for analytical measurements needs several requirements to be addressed. These would primarily involve the need for mass production of the sensor, therefore providing a sensor easy to fabricate, inexpensive and reproducible in substantial quantities. Low cost electrochemical sensors which would easily be produced and disposed after each use has being proposed before (Cardosi and Turner, 1990). This type of sensors normally is based on screen-printed electrodes (Bergveld and Turner, 1993). However, the majority of electrochemical units assembled in research laboratories are not suitable for cost-effective mass production (Gyurcsányi *et al*., 2001). Screen printed electrodes (SPE) have found application in the production of chemical sensor arrays and amperometric biosensors (Bilitewski *et al*., 1991; Cagnini *et al*. 1995; Gilmartin and Hart, 1994; Hampp *et al*., 1994; Hart *et al*., 1996; Newman *et al*., 1995; Schmidt *et al*., 1994; Wang *et al*., 1995; White *et al*., 1994a, b). Variety of commercial biosensors such as ExacTech glucose pen has being fabricated using screen printing.

Sensor microfabrication technologies such as thick-film and thin film lithography fulfil the high volume production of microcircuits suitable for electrochemical sensing devices as "user-friendly" approaches. These devices involve planar working and reference electrodes which are printed on plastic or silicon substrates. This design avoids the use of traditional bulky electrodes or the "beaker-type" ones. A droplet is only required to assess an electrochemical current using the appropriate electrode configurations (Wang *et al*., 1996; 1998).

Screen-printing was used at first in the graphics industry. Ever since the 1950s the technique has been developed in order to cultivate a conducting, resistive and insulating paste that would be employed later on for the production of miniaturised hybrid circuitries. In screen-printing, viscous compounds are deposited as a film on the plastic, ceramics, glass etc. surface. This requires usage of a paste with controlled and well-characterised properties in order to achieve similar characteristics of electrodes each time

(Bilitewski *et al*., 1991; Goldberg *et al*., 1994). Thick film technology permits the construction of robust and planar electrodes. Dimensions of these electrode structures vary from 50 μm that require microlithographic processes, to 75-100 μm that could be constructed with screen-printing technology. The technology for the construction of these types of electrodes is a well established procedure used for the large-scale production of electronic components and sensors. Printing technology offers advantages such as flexibility of design (several ready made configurations exist), choice of materials (deposition of metal conductors, dielectric insulators and polymeric pastes), relatively easy integration with external electronic circuits, mass production and low cost fabrication, integration with liquid handling features (e.g. flow injection systems), and automation in the use of printing fabrication. There is also the possibility of miniaturised systems or even the possibility of using membranes and porous material that would enhance the electrochemical activity of the produced electrodes. The main trend in this type of technology is the integration of the working sensor with a reference and a counter electrode on one device.

Unfortunately, like most techniques, thick film technology has a few drawbacks. The majority of the problems encountered with this technique include the manufacturing processes, particularly the compatibility of the printed materials. The other major problem would be fabrication process, which often requires high temperature and a relatively long time. There is also problem with evaporation of the solvents in the formation of the paste (Galan *et al*., 1995).These problems together often result in low reproducibility of the sensors.

To print electrodes, a planar substrate has been used along with polymer based inks. This is followed by a thermal curing. The development of the screen-printed electrodes is commonly achieved by the sequential deposition of these inks on a supporting layer. There is a wide range of inks and supporting materials. Commercial carbon, platinum, gold and silver/silver chloride for reference electrodes (Wang *et al*., 1996; Newman *et al*, 1995; http://www.bvt.cz).

Carbon or graphite materials are the most commonly used as they are associated with several benefits. Among them is the electrochemical inertness over a wide range of potentials, hydrogen and oxygen evolution overvoltages, low background currents and

minimisation of noise, and high electrical conductivity (Osborne *et al*., 1996). Carbon inks contain carbon black, graphite particles, polymer binders and other relevant additives (suitable for printing and adhesion tasks) with the exact ink or paste formulations provided by the manufacturers as exclusive proprietary information (Wang *et al*, 1996). Therefore during practical development work it is necessary to investigate further the behaviour of these materials and the nature of the specific reactions at the electrode surfaces, which could provide important data for practical electrochemical performance of the electrodes. Electron transfer reactivity is affected by the type, size and loading of the graphite particles in the ink used. Characterisation of screen-printed electrodes has being extensively studied in previous publications (Osborne *et al*, 1996; Wang *et al*., 1996; Cui *et al*., 2001; Grennan *et al*., 2001; Morrin *et al*., 2003). The performance of thick-film sensors using four commercial carbon inks has being extensively studied by Wang *et al*. (1998). The main objective of this work was the comparison of the electrochemical behaviour of commercial inks from Acheson (U.S.A.), Gwent (U.K.), Dupont (U.S.A.) and Ercon (U.S.A.). Attention was paid mainly to background currents and the electrical activity of redox systems. Screen printed electrodes are the major type of electrodes within commercial sensors used (SPCEs). The SPCEs exhibited qualities such as peak separation, hydrogen and oxygen overvoltage for redox systems. The general view is that different commercial compositions and preparations of SPCEs would show different electrochemical activity and due to this every practical development will require optimisation of the electrodes performance.

Glassy carbon electrodes have also being studied and compared with SPCE electrodes (Grennan *et al*., 2001). The comparison was performed by using a ferrocyanide redox system. Results indicated a peak separation (ΔE_p) of glassy carbon electrodes (66-76 mV) lower that the peak separation of the SPCEs (100-120 mV), at a scan rate between 10 to 100 mV s^{-1}. Eventually, the heterogeneous electron transfer for the ferrocyanide was $k^o = 2.2 \times 10^{-3}$ cm s^{-1} for the SPCEs and $k^o = 1.2 \times 10^{-2}$ cm s^{-1} and this difference was attributed to the presence of polymeric binders in the carbon in that could inhibit the electron transfer.

Furthermore, potassium hexacyanoferrate (III) has been studied by Osborne *et al*. (1996) using cyclic voltammetry. The results obtained were attributed to the surface properties of the printed electrode. Further comparison of commercially available SPCEs

and electrodes fabricated *in-situ* from various commercial inks, was performed by Morrin *et al*. (2003) in order to find the most suitable electrodes for amperometric sensor application. The commercially available electrodes have yielded a very weak reversibility for ferricyanide and ferrocyanide couple with ΔE_p values varying from 471 ± 56 mV to 416 ± 37 mV, respectively. The difference between the values is attributed to poor charge transfer at the electrode surface. In addition, the conducting parts of the sensors had resistive properties highly likely to be a limiting factor.

Thick-film technology has been used also for manufacturing of reference electrodes. Thus reference electrodes containing silver/silver chloride have also being reported by Desmond *et al*. (1996; 1997; 1998) and Erlenkötter *et al*. (2000). The potential of silver/silver chloride (Ag/AgCl) electrodes has being studied over time and results indicated a good reproducibility and stability, despite the fact that this paste in order to function properly requires a constant supply of chloride ions.

The work presented in this chapter describes fabrication and characterisation of the screen printed electrodes for the application in an electrochemical sensor for the detection of hydrogen peroxide. These sensors were used later in an attempt to monitor the process of photosystem inhibition by herbicides.

Electrochemistry has been used for the characterisation of SPEs, produced from Cranfield and elsewhere. Among these were several different types of electrodes provided by Krejci Engineering Ltd (Czech Republic). Some characteristics of these electrodes were provided by manufacturers and could be found on www.bvt.cz.

3.2 EXPERIMENTAL

3.2.1 Materials

Graphite-based ink was type 423SS (Acheson Electrodag Colloids, U.K.), the silver/silver chloride (Ag/AgCl) type Electrodag S038SS (MCA services, Cambridgeshire, U.K.), the protective polymer (insulating ink) type 242SB (Agmet ESL, Reading, U.K.), and the solvent (thinner) for the ink 242SB was type 402 (Agmet ESL, Reading, U.K.). The inks were printed on a “Melinex” polyester sheets (228 x 350 mm),

obtained from Cadillac Plastic (Swindon, U.K.). The equipment used for the screen-printing is a DEK 248 screen printer machine (Figure 3.1) which is available at the Silsoe campus and was manufactured to specification by DEK Precision Screen Division (Weymouth, U.K.). Electrodes provided by Krejci (AC1.W2.RS) are amperometric electrodes on an alumina ceramic base using pure platinum as a working electrode material. More specifically they have 100% Pt as working surface, Ag/Pd (98/2%) as the reference surface, Pt (100%) as the auxiliary material and pure Ag as the conducting path.

The reagents were of analytical grade unless stated otherwise. Water purified by, reverse osmosis water was used for cleaning of the glassware and dissolution of the compounds. Potassium chloride (KCl), potassium ferrocyanide ($K_4Fe(CN)_6 \cdot 3\ H_2O$), were from Sigma. Di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4) and sodium dihydrogen orthophosphate anhydrous (NaH_2PO_4) were purchased from BDH Laboratory Supplies, Merk Eurolab (Darmstadt, Germany) and used for the preparation of phosphate buffer solution.

Figure 3.1: A DEK 248 screen printer machine available at Cranfield University.

3.2.2 Equipments and techniques used

The techniques used for the analysis and evaluation of the analyte are cyclic voltammetry and amperometry. All measurements were performed with an Autolab Electrochemical Analyser with the general purpose electrochemical software GPES 4

(Ecochemie, Utrecht, Netherlands). Data from both electrochemical applications were processed using Microsoft Excel. Screen printed electrodes were connected to an IDE edge connector from Maplin Electronics plc (Wombwell, South Yorkshire, UK) insulated at one side for a tighter connection. A specially adapted electrical edge connector provided by the electrochemistry laboratory (Silsoe, Cranfield University) was used for the connection of the electrodes to the electrochemical analyser. Glassy carbon electrodes and platinum wire electrodes were obtained from Bioanalytical Systems Ltd. (Cheshire, U.K.). All experiments were performed at room temperature between 18 ^{o}C to 20 ^{o}C.

Chronoamperometry with an interval time greater than 0.1 sec. was used and current was defined as a function of time. In chronoamperometry the potential is controlled at a value sufficient to react immediately with sample molecule that diffuses to the surface. In case of a disconnection the present scheme restores the initial condition (Kissinger and Heineman, 1984).

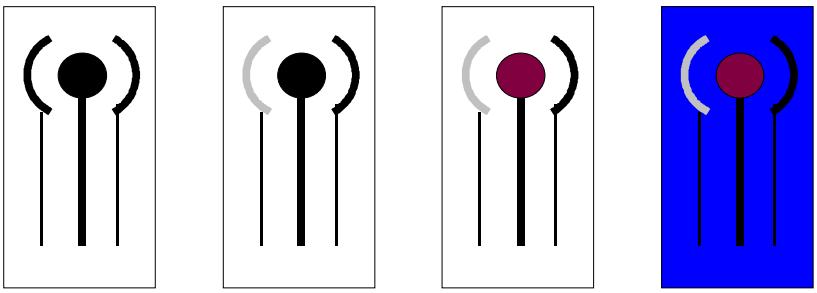
3.2.3 Safety considerations

COSHH forms were filled prior to any commencement of laboratory work (Control of Substances Hazardous to Health). For all inks used during screen-printing, disposal requirements were displayed on the fume cupboard in the fabrication laboratory (Silsoe, Cranfield University). The recommendations for the identification, disposal and emergency policies for the hazardous substances were adhered to at all times.

3.2.4 Screen-printing electrodes for thin-film technology

To print electrodes, a plastic substrate has been used along with polymer based inks. The development of the screen-printed electrodes requires approximately a week. An unmodified graphite electrode requires three steps (1,2, and 4 in Figure 3.2) and a metallised electrode required four steps. The polymer based inks along with the plastic substrate require drying at temperatures from 120^{0} C to 300^{0} C. Where it was not possible to attain such temperatures, drying could be achieved by allowing the inks to dry overnight at room temperature (usually 18^{0} C).

The relevant paste is pushed through a mesh on plastic substrate. Usually the distance between the threads of the mesh is three times than the particle size of the ink and the minimum line printed is approximately three times the mesh thread diameter. The mesh is actually stretched over a rigid aluminium frame. The frame is securely placed on a DEK 248 screen printer machine and covered with sufficient amount of ink. The substrate is held on a moving platform that is moved under the screen which is lowered, but is not actually touching the substrate (off-contact) because the edges have been slightly built up. A firmer pressure is required to be forced on the squeegee to push the ink through the open mesh to the substrate. As the squeegee passes over the screen, the substrate moves downwards and out of the machine. It is possible to produce several printed sheets before it is necessary to apply more ink on the screen. This is happening when printability is poor and usually there is some loss of electrodes in at least two sheets.



(1) (2) (3) (4)

Figure 3.2: Screen-printing electrodes: 4 steps.

(1) Carbon ink on plastic substrate.

(2) Ag/AgCl reference electrode on carbon.

(3) Modified working electrode (Rhodonised in this study).

(4) Insulating ink.

Screen-printed electrodes were printed onto a 250 μm thick "Melines" polyester sheet (229 mm x 305 mm) enabling a production of 60 to 100 electrodes per sheet (Figure 3.3). The different ink layers including the counter electrode, the reference electrode, the working electrode, and finally the insulating layer were printed one after the other. The first layer was printed is the carbon ink where it serves as working surface and also as the conducting path, and required drying at 60^0C. The second layer is the Ag/AgCl where it serves as the reference electrode and requires similar drying temperature as the carbon layer.

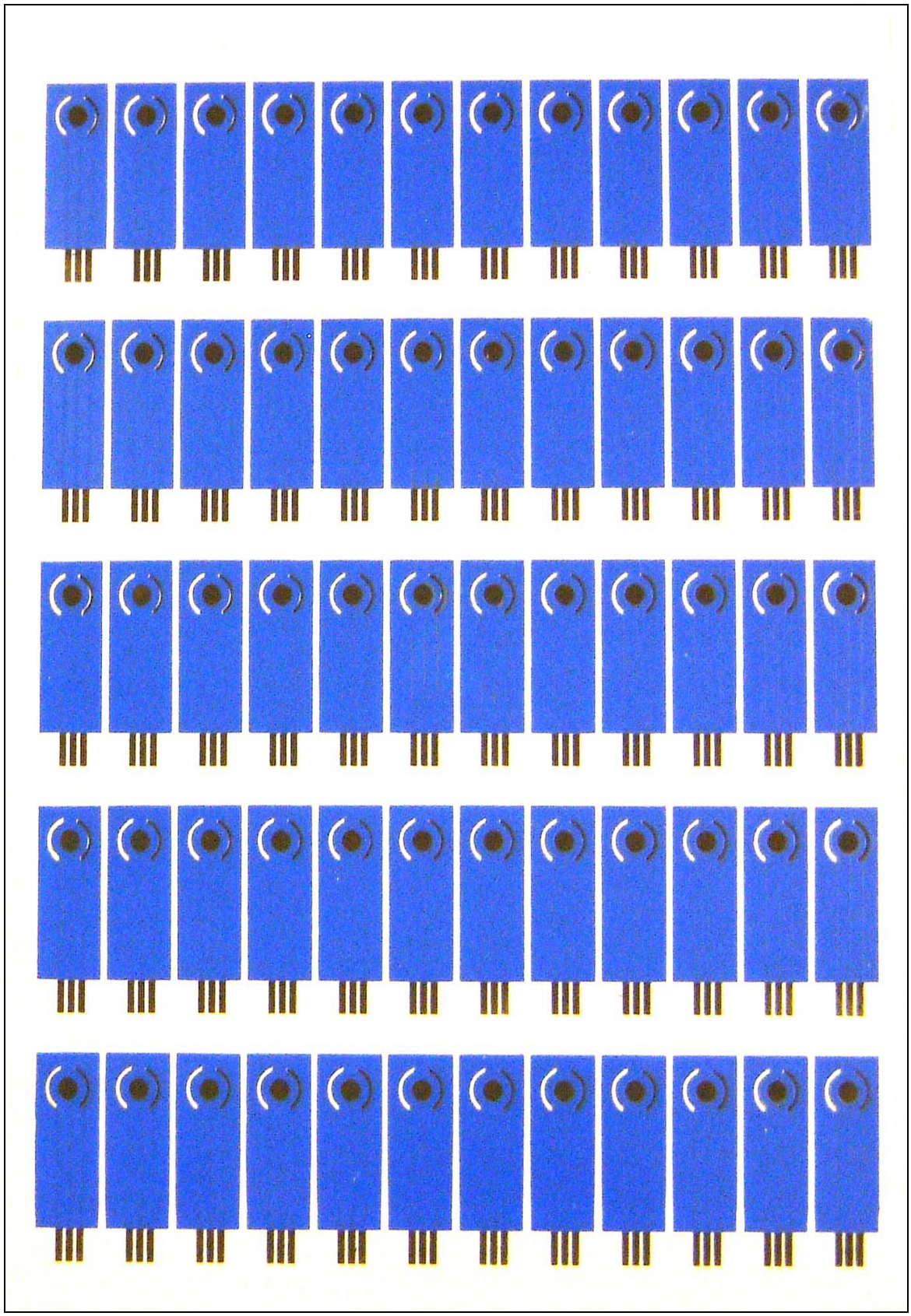


Figure 3.3: Carbon electrodes configuration. The representation of 60 electrodes per sheet.

To isolate all parts an insulation ink was printed on top of all layers leaving uncovered the working, reference, and conducting path circuitries. Insulation ink requires drying at a temperature of 120 ^{0}C. The most important steps after finding the appropriate Mylar flap for the needed type of electrodes is to edit the parameters and adjust the print gap after changing the print mode from double squeegee to print/flood. Deposition of ink on the plastic substrate was achieved by forcing the paste through the open mesh of the Mylar flap which a screen is having a net mounted on an aluminium frame. A silicone spatula (squeegee) was used for spreading the ink uniformly. The lay-out drawn on the open meshes of the screen produces a substrate yielding simple or even complex circuits. As a guideline, the distance between the screen and the substrate (print gap) can be gauged using the width of the screen multiplied by: 0.004 for stainless steel mesh, 0.006 for polyester, or 0.01 for Nylon. The parameters which were set on the DEK 248 screen printer machine are given in Table 3.1.

Table 3.1: Parameters set on DEK machine for screen-printing.

Print parameters	**Settings**
Print mode	Print/Flood
Squeegee pressure	4kg
Print gap	2.1 mm (depending on substrate)
Forward carrier speed	50 mm.s^{-1}
Reverse carrier speed	50 mm.s^{-1}
Front limit	38 mm
Rear limit	400 mm
Separation speed	70%

3.2.5 Screen-printed electrodes of thick film technology

One type of electrode used in this work is AC1.W2.RS provided by Krejci Engineering (Figure 3.4). This is a thick film sensor, printed on a base made from Alumina Ceramic (A_2O_3 -96%). This type of electrode has as a working surface 100 % platinum, as a reference 60/40 % Ag/AgCl and as auxiliary a 100 % platinum. The

connection of the sensor occurs through silver conducting paths which are able to minimise the potential difference between the working electrode and the potential actually applied on the system. They have a mass of 0.4 g, a width of 7 mm, length of 26 mm, and a thickness of 0.65 mm. Other electrodes also tested here were L5, L4, and L1, where L5 had the same material as AC1.W2.RS, but had a membrane of acetate cellulose, L4 had basic graphite paste as substrate and acetate cellulose without any metallised materials that could enhance the potential of the measurements, and L1 were the same as AC1.W2.RS but having graphite paste printing on top of the platinum. All these electrodes were tested and quantified in comparison to the electrodes fabricated at Cranfield University and overall results were obtained in terms of sensitivity and selectivity of each type.

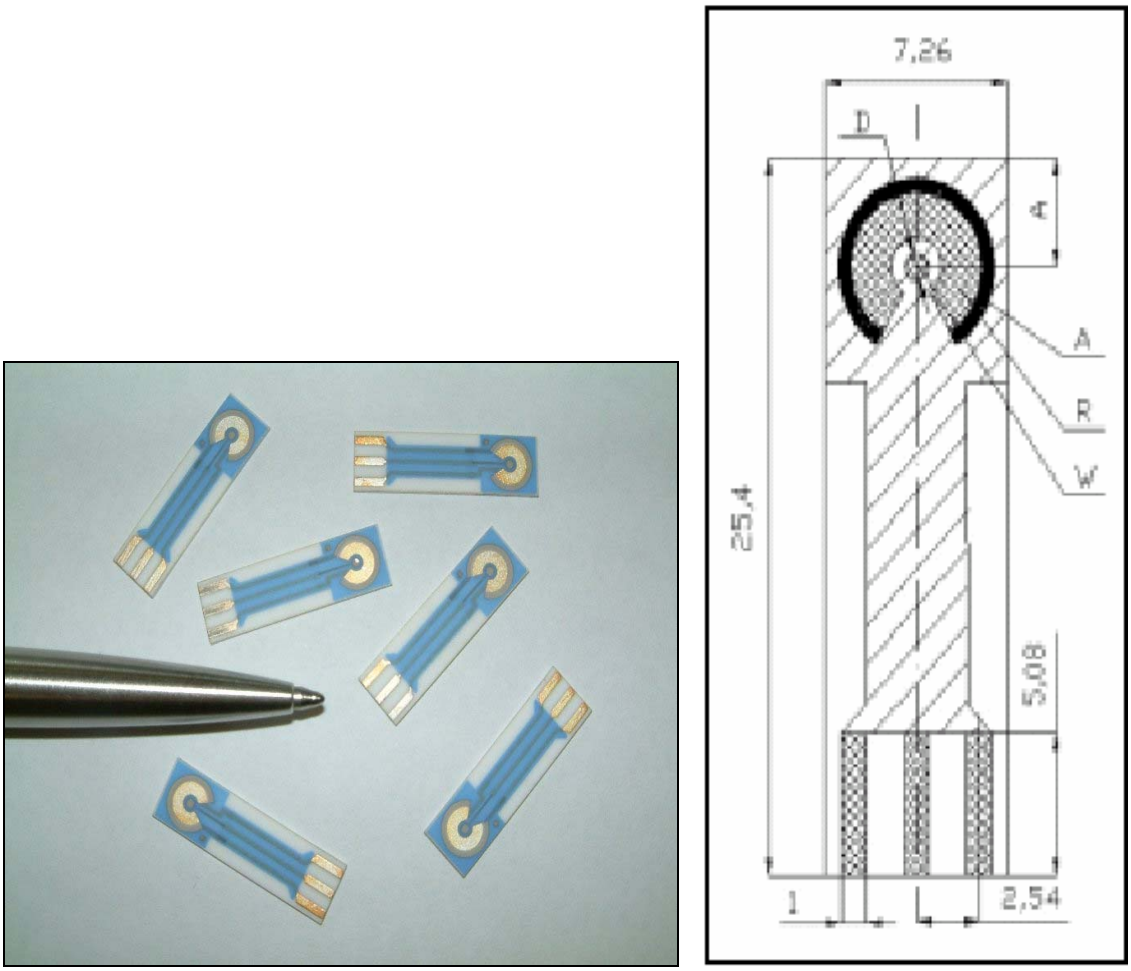


Figure 3.4: Substrates for amperometric sensors type AC1.W2.RS provided by Krejci engineering www.bvt.cz.

3.2.6 Modified screen-printed electrodes

Carbon material is considered to be one of the most important anode materials. This is due to the superiority of the carbon in terms of high capacity, low irreversible capacity, low potential profile, and low cost (Gyurcsányi *et al*, 2001). However, its disadvantages such as high sensitivity to electrolytes and easy exfoliation have led to a dynamic investigation on the surface modification of the graphite.

In the most common configurations of sensing strips, the working electrode is produced of carbon (graphite) ink, screen-printed on separate strips with various polymeric or ceramic supports, or on the same strip along with other electrodes in order to make a sufficient electrochemical cell. Recently carbon nanotubes, discovered in 1991 by Iijima have been printed onto the electrode surface (Iijima, 1991). Apparently the performance of these electrodes was improved. Several other forms of carbon modification included incorporation of metal nanoparticles and integration with polymers.

3.2.6.1 Rhodonised screen-printed electrodes

The modification of electrodes with metal nanoparticles allows the tailoring of surfaces with additional catalytic properties which might exclude need in enzymes for generating sensor response. Rhodonised carbon electrodes were prepared by printing a rhodonised ink layer over the graphite base layer of the working surface of the electrode. The rhodonised ink contained 5% rhodium (MCA4a powder). The way that the electrocatalyst was incorporated into the electrode surface was by diluting the MCA4a powder with v/w 2-butoxyethyl acetate (Newman *et al*, 1995) (Figure 3.5). To prepare this mixture 10 g of Electrodag 423 SS was mixed with 5 g of rhodonised carbon powder MCA4a. The mixture was stirred thoroughly by with a plastic spatula for 1 minute. When the mixture became relatively homogeneous, 50 % v/w (7.5 ml) of 2-butoxyethyl acetate was added to improve the printability of the paste.

Figure 3.5: Electrodes with incorporated MCA4a ink (rhodonised).

3.2.6.2 Polymer-modified screen-printed electrodes

Polymer-modified electrodes were prepared in the same way by mixing the already available graphite ink with the polymer particles. To homogenise the mixture, a few drops of the solvent (thinner) for the insulating ink was also added. The mixture was then screen-printed over the already printed graphite substrate.

The affinity polymer prepared for mixing with graphite consisted of 10 mmol (1.98 g) of ethylene glycol dimethacrylate, 1 mmol (134 mg) of o-phthalic dialdehyde (OPA), 2 mmol (148 mg) of allyl mercaptan (AM) and acetonitrile (2 ml). The solution of the monomer was purged with nitrogen and the polymerisation was initiated with the addition of 50 mg azobis (isobutyronitrile) (AIBN) and left overnight, heating at +80 ^{o}C. Polymer was ground and washed with methanol and the fraction of particles with size 38-63 microns was collected by sieving (Piletska *et al*., 2000). In order to prepare the polymer/graphite electrodes, 20 g of carbon ink were used and mixed with 3.3 g of polymer after sieving through a mesh of 25-75 μm. The graphite/polymer electrodes were

used for enzyme assay measurements (results are shown in chapter 4 section 4.3.3 and 4.3.4) along with mediated hydrogen peroxide detection.

3.2.7 Electrochemical measurements

Electrochemical measurements for the characterisation of the electrodes were performed using cyclic voltammetry. All measurements were performed at ambient temperature.

3.2.7.1 Cyclic Voltammetry

Cyclic voltammetry was used to characterise the working surface of the screen-printed carbon electrodes using as an electroactive marker, potassium ferrocyanide. Data were compared to these obtained with glassy carbon electrode. Electroactivity of the working surface was determined as well as the electrochemical reversibility of potassium ferrocyanide on the electrode, which is a standard approach for those working on the development of a biosensor with this technique. Scan for potassium ferrocyanide was set to cycle from -1.5 to 1.5 V relative to Ag/AgCl reference electrode. Stock solutions of 50 mM potassium ferrocyanide were prepared in 0.1 M KCl and a phosphate buffer of pH 7.4 (40 mM). Measurements were performed with electrodes being in a vertical position, dipped in a beaker with 10 ml buffer solution. The electrochemical behaviour of potassium ferrocyanide was tested at different scan rates: 20 mV, 50 mV and 80 mV. Before measurements glassy carbon electrodes were polished with alumina powder (0.3 μm) on a polishing cloth, and then rinsed with reverse-osmosis water.

3.2.7.2 Miscellaneous measurements.

Resistivity of the working surface of the electrodes was also measured. Normally electrical resistance plays a small role in the actual variations of the applied potential. Higher values of resistivity, would however result in higher deviations of the applied potential, affecting actual electrochemical measurements. Readings were carried out using a digital 50,000- count TRMS multimeters that provide measurements for V_{AC}, $_{AC + DC}$, I_{AC}, $_{AC + DC}$, Ω, continuity, diode test, capacitance, frequency, temperature, network monitoring, dB.

3.2.8 Analysis

All experiments were carried out using three or more screen-printed electrodes for the same measurements. Each measurement was saved and treated in order to obtain an error value that would be represented by standard deviation. Relative standard deviation values were provided for each analytical experiment and were calculated using GPIS software (version 4) with Microsoft Excel office software.

3.3 CHARACTERISATION OF SCREEN-PRINTED ELECTRODES

3.3.1. Electrochemical characterisation of SPEs

Cyclic voltammetry enables the search of redox potential(s), which would characterise the electrodes (Kissinger and Heinemann, 1984). In order to characterise the electrodes, the electrochemical behaviour of potassium ferrocyanide was studied since its electrochemical behaviour is well known. During a voltammetric scan, the potential applied to the working electrode becomes sufficiently positive at a certain value and causes analyte oxidation. An anodic current is observed at that point which increases rapidly until the oxidised species fall to zero. During the reduction of the electrode immersed in a solution, the potential scan is switched to negative values and the accumulated oxidised species on the electrode surface are reduced. One cycle is complete when the now reduced species are depleted in order to cause the current to peak and then to decrease.

The CVs obtained for potassium ferrocyanide on graphite electrodes (Figure 3.6) are relatively similar to the ones obtained on glassy carbon ones (Figure 3.8) and to the ones observed from carbon/polymer electrode (Figure 3.7). As it is observed from both electrode types, the lower the scan rate is, the higher is the separation between the anodic and the cathodic peaks. Furthermore, oxidation/reduction peaks are more defined when the low scan rates are used.

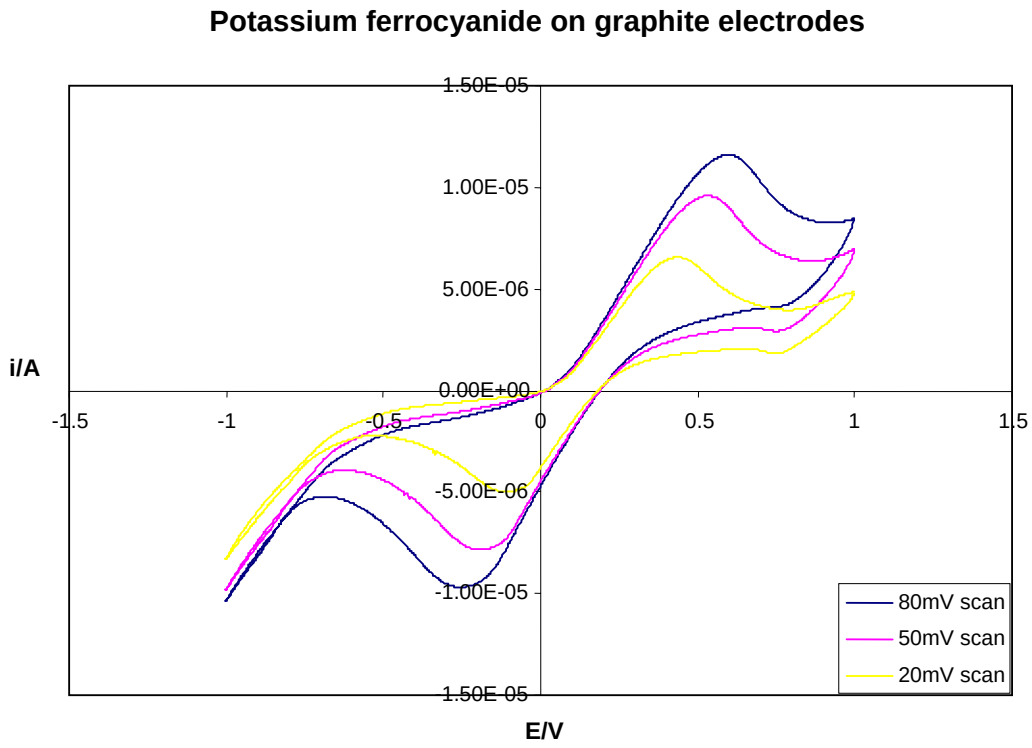


Figure 3.6: Cyclic voltammetry of 50mM potassium ferrocyanide at three scan rates 20mV, 50mV, and 80mV on a screen-printed carbon electrode.

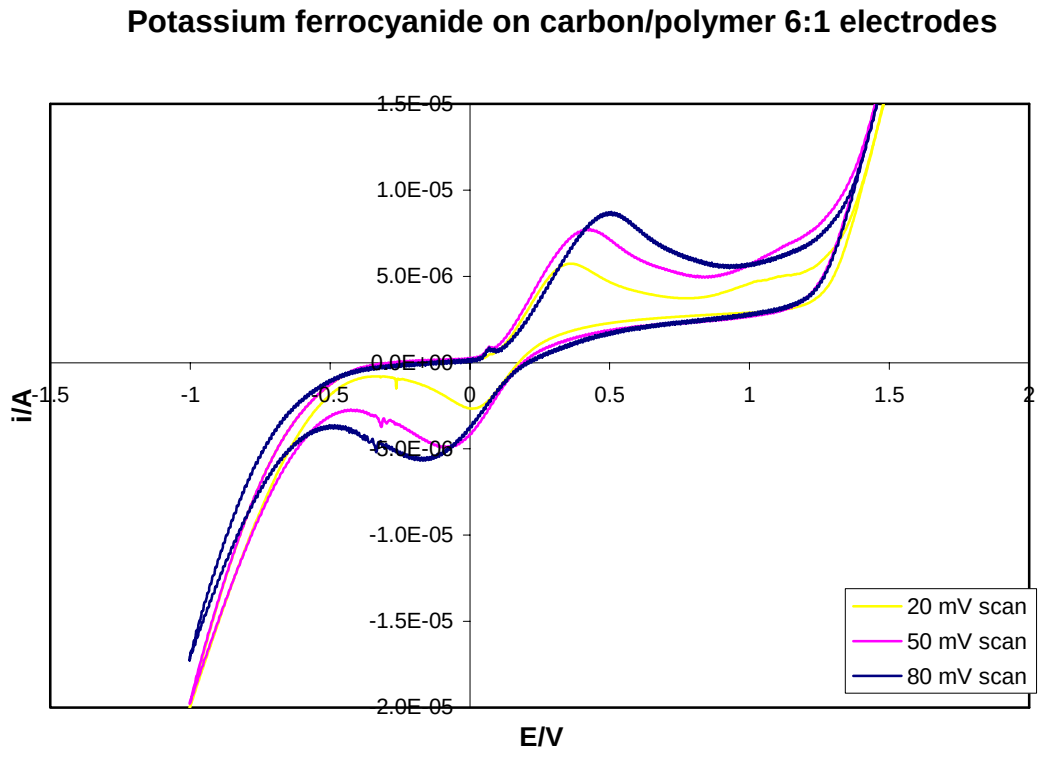


Figure 3.7: Cyclic voltammetry of 50mM potassium ferrocyanide at three scan rates 20mV, 50mV, and 80mV on a carbon/polymer electrode.

At the scan rate of 50 mV the peak separation for carbon electrodes was in the area of 450 mV and for carbon/polymer in the area of 400 mV compared to the glassy carbon ones (63 mV). Usually the separation from the anodic to the cathodic peaks for a reversible one-electron transfer reaction is in the area of 59 mV. All electrodes gave a peak separation greater than the latter and this could be explained by several reasons. The decrease in electron transfer reactivity for the SPCEs could be due to the graphite particles homogeneity and possibly due to presence of a binding substance in the graphite ink that would affect the redox activity of the electrode and give results slightly different to the glassy carbon electrodes. This shift in peak separation is a result of uncompensated bulk resistances in the thick film and charge transfer to the dry electrode (Wang *et al* 1996, Wang *et al*., 1998). The difference between the carbon electrodes (Figure 3.6) and the carbon/polymer ones (Figure 3.7) could be attributed to the polymer mixed in the carbon paste. To identify these electrode surfaces in detail, electron scanning microscopy has been applied and further discussed in Section 3.3.2.2.

Additionally, in all figures there is an increase in the peak separation depending on the scan rate that suggests that screen-printed electrodes and glassy carbon are quasi-reversible.

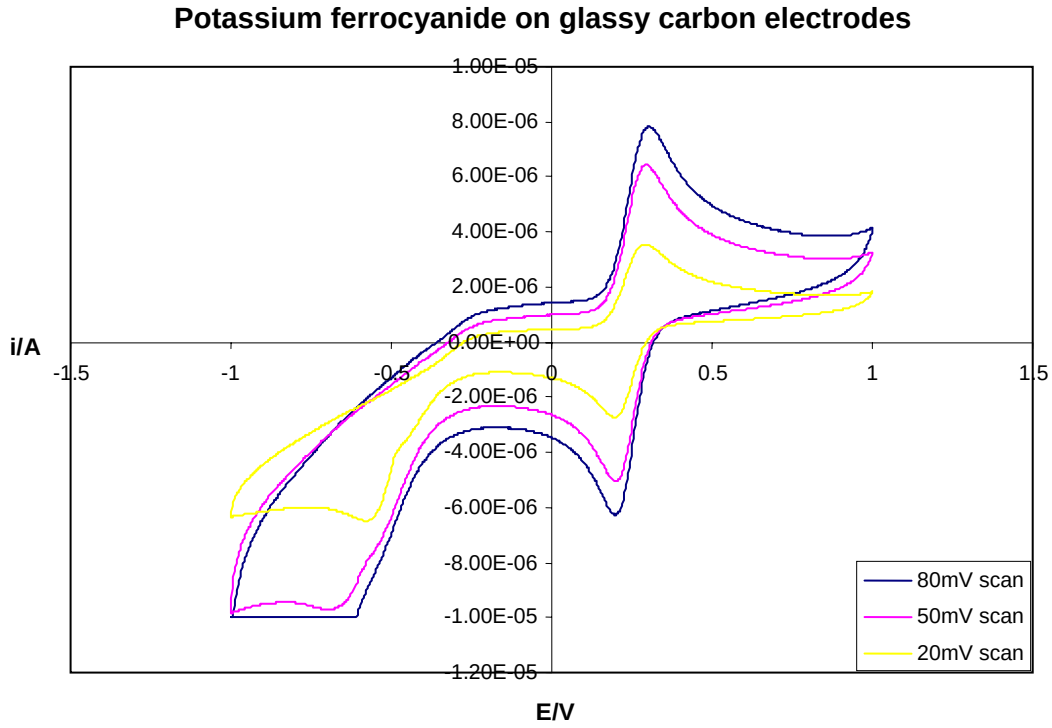


Figure 3.8: Cyclic voltammetry of 50mM potassium ferrocyanide at three scan rates 20mV, 50mV, and 80mV on glassy carbon electrode.

3.3.2. Surface characterisation by scanning electron microscopy

Carbon and polymer/graphite electrodes were characterised by using scanning electron microscopy (SEM). Electron microscopes have been developed due to the insufficiency of Light Microscopes which are limited to physical parameters of light ranging from 500x to 1000x magnification and a resolution of 0.2 μm. In early 1930's there was a scientific urge to see fine details of the interior structure of organic cells such as nucleus, mitochondria, etc. Unfortunately, in order to observe such structures, a 10,000x magnification was required. In the first transmission electron microscope (TEM) the light beam was replaced by a focused beam of electrons. This had helped to "see through" the specimen. In 1942, the first SEM appeared that became commercially available in 1965. The SEM differs from the TEM by using a scanning beam of electrons across the sample.

Electron microscopes are scientific instruments that use a high energy electrons beam in order to examine objects at high magnification. The information drawn out of the received pictures, provide information on the topography, morphology, composition and crystallography of the target. The first parameter of the above mentioned involve the surface features of the objects, and these features are directly related to the materials properties. Morphology is directly related to the shape and size of the particles. Finally, last set of information that could be withdrawn from the electron scanning microscopy data would be the elements and compounds that the object is composed of and also the way the atoms are arranged (Hart, 1976).

3.3.2.1 Preparation of electrodes for SEM analysis

The working surface of the screen-printed electrodes (both carbon and polymer/graphite) were cut and placed onto round holders which were later on used in the SEM instrument as a base. Surfaces used in electron scanning microscopy need to be conductive. Therefore, apart from metals all other objects required to be observed through this technique need to be covered with a conductive later. An efficient approach would be to coat the sample areas with conducting surfaces such as gold, chromium or silver. The method used in this work was gold evaporation. An Edwards coating system E306A was used for evaporating gold on the screen-printed disks. The samples were placed at metal coating prisms which have the relevant values for gold deposition (density of 19.3, acoustic impedance of 23.17, and tooling of 1.0). The disks were glued onto the holder prior to gold evaporation. For electron scanning microscopy the instrument used was an ABT-55 SEM. Each sample was analysed repetitively three times to avoid possible artefacts.

3.3.2.2 Visualisation of electrode surface

The paste of carbon consists of grains of active material and the binding material. The grains of active material are usually very small (1 μm) and a large numbers are already included in the commercial paste. In common pastes, the proportion of the binding material is relatively small. Commercial pastes are generally hydrophobic surfaces, resistant to water and therefore organic chemicals would penetrate directly to the graphite grains. Activity of the surface would mainly be influenced by the morphology,

meaning that the homogeneity of the binding materials would play an important role to the properties of the paste.

In the case of polymer/graphite electrodes, the polymer grains were mixed in a sufficient way in order to incorporate them with the binding materials of the carbon paste. The carbon paste shows a relatively homogeneous surface (Figure 3.9). There is a minimal amount of cavities present which is normal, as these exist already in the commercial paste. The picture of polymer/graphite electrodes shows more advanced structure as compared with graphite electrodes (Figure 3.10). The pictures collected from both materials were performed at values of HV (High Vacuum) of 8 KV.

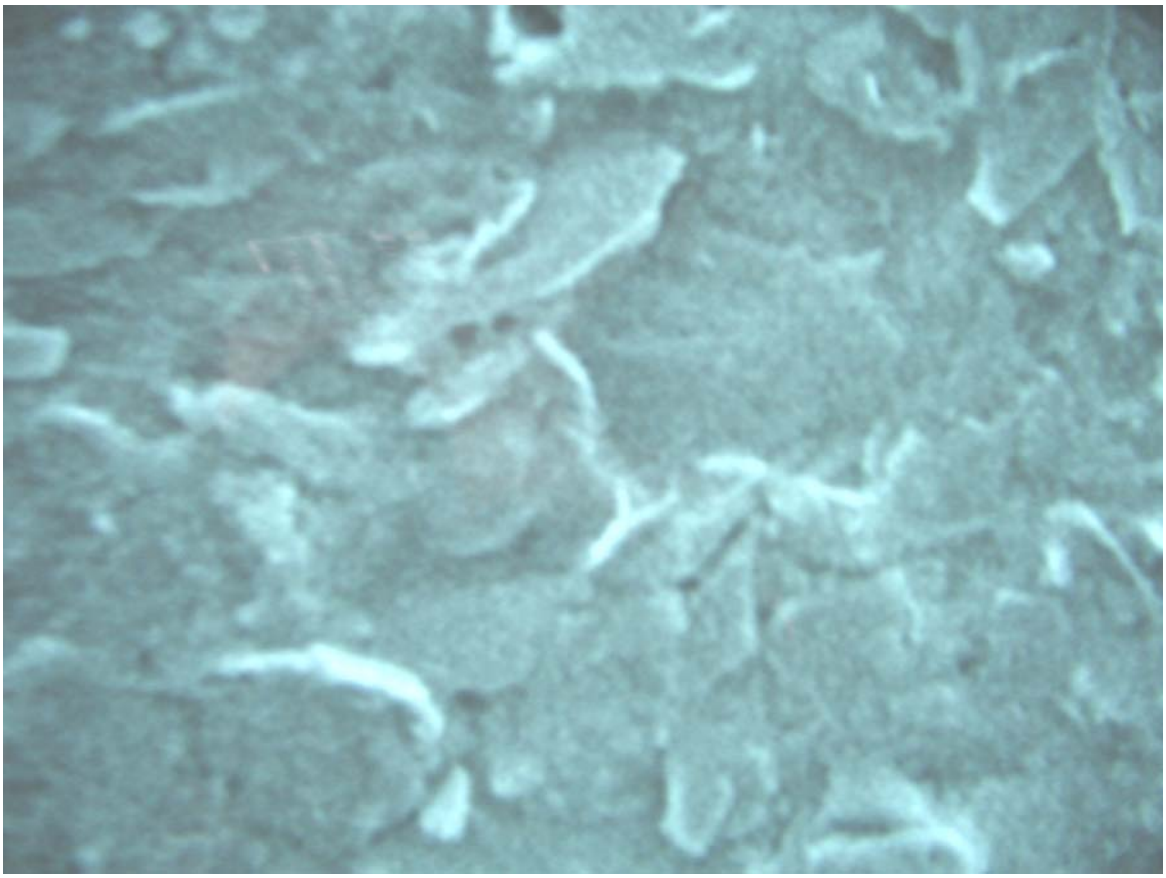


Figure 3.9: SEM picture of carbon electrodes.

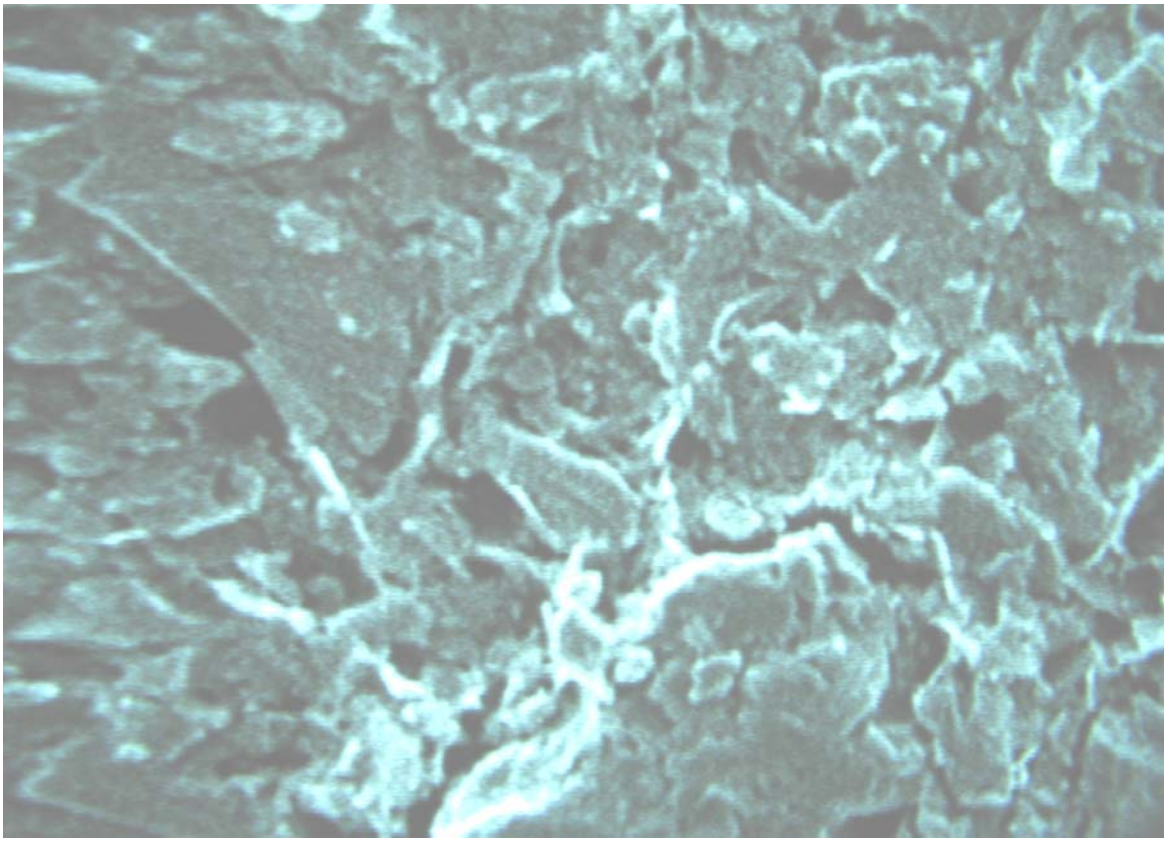


Figure 3.10: SEM picture of polymer/graphite electrodes.

3.4 CONCLUSIONS

In conclusion, a set of screen printed electrodes have been prepared using DEK 248 screen printer. The use of screen printing was succesful allowing production of hundreds and even up to thousands of electrodes. Electrochemical testing with potassium ferrocyanide has been used to characterise efficiency of electrochemical performance of prepared electrodes. The three-electrode system has been developed for later use in amperometric detection of H_2O_2 and for the detection of herbicides.

The graphite electrodes were also modified with rhodium and with affinity polymer in attempt to enhance the sensitivity of electrodes. Both modified electrodes have shown good electrochemical performance similar to non-modified samples. The higher sensitivity of the polymer-modified electrode resulted from its more advanced surface as can be seen from the SEM photograph. These electrodes along with the standard carbon ones were further studied in the following chapter on their ability to detect low concentrations of hydrogen peroxide.

CHAPTER 4. MOLECULARLY IMPRINTED POLYMER MEMBRANES

4.1 INTRODUCTION

Molecular recognition involves the selection and binding of biological and synthetic molecules. It is considered to be of universal interest as it is the basis of biological processes like the translation and transcription of the genetic code, substrate-enzyme reactions and ligand-receptor binding. The principles involved in molecular recognition exhibited by natural molecules have been effectively transferred into synthetic receptors (Lehn J.M., 1988; Cram D.J., 1992).

The practical applications of natural receptors are limited by the instability of the biomolecules, and their sensitivity to physical and chemical influences which could lead to low specificity of the synthesised materials. In 1972, Wulff and Sarhan introduced a new methodology for the development of synthetic materials which is based on the preparation of specific receptors sites in cross-linked polymers. This is generally known as imprinting polymerisation and includes the formation of a monomer-template complex and its polymerisation in the presence of cross-linking agents. During the polymerisation step, the fixation of the monomer-template complex in a rigid polymer network formed during the polymerisation process produces recognition sites containing polymeric functionality positioned to complement those of the template molecule. After polymerisation and extraction where the template is removed, results in cavities which posses the shape of functional group which corresponds to the template (Figure 4.1). This procedure allows such synthetic polymers to mimic biological receptors in specificity and affinity. (Ramstrom *et al*., 1993; Piletsky *et al*., 1998).

Typical molecularly imprinted polymers (MIPs) are prepared by bulk polymerisation. This is followed by washing and grinding of the appropriate polymers with the removal of the template used during the imprinting process, which yields polymer particles having receptor sites on their surfaces. Alternatively, MIPs could be synthesised on preformed non-porous or porous particles or films. Molecular imprinting is a generic technology and can be used in a variety of applications such as: (i) chromatography (e.g. chiral separation); (ii) solid phase extraction; (iii) drug/fragrance release matrices; (iv) adsorbents for clinical and environmental applications; (v) sensors

and multisensors for environmental, clinical diagnostics and extreme environments. The advantages of MIPs for such applications are their selectivity, the simplicity of their synthesis and their ability to detect low-molecular weight compounds (Siemann *et al.*, 1996) and their relatively low cost. Furthermore, their thermal and mechanical stability allows them to be applied especially as stationary-phase material in selective chromatographic columns and/or substitutes of antibodies in competitive binding assays (Piletsky *et al.*, 1996; Vlatakis *et al.*, 1993).

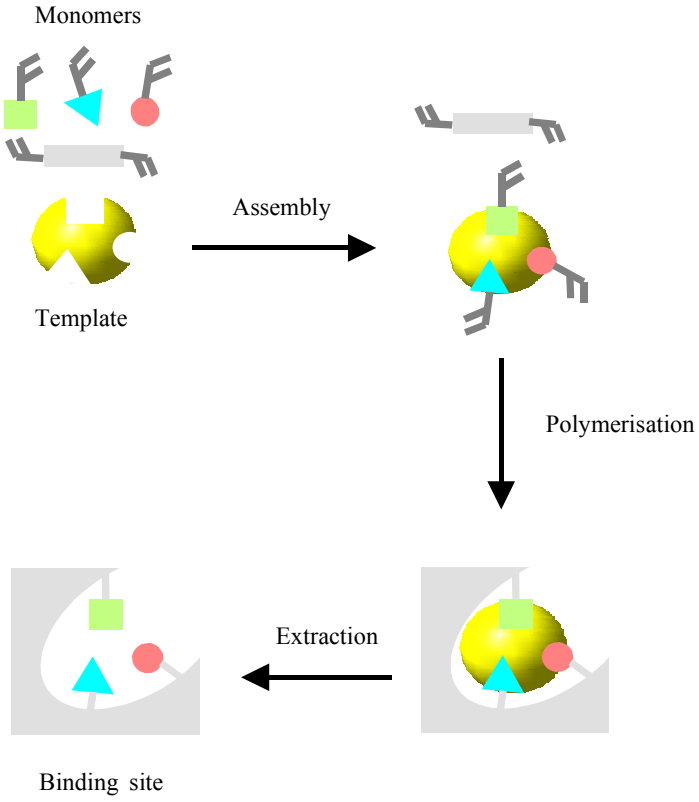


Figure 4.1: Schematic of imprinting polymerisation. The appropriate functional groups bind to the polymerisable template molecule. Copolymerisation of this monomer-template complex with a crosslinker, and subsequent extraction of the template yields a molecular imprinted polymer (MIP). This leads to a binding site containing microcavities with specifically oriented functional groups, complementary to the shape and function of the template.

Recently MIPs have been used in the form of free standing membranes. This structure allows direct applications to sensor technology (Piletsky *et al*., 1995; Piletsky *et al*., 1998; Piletsky *et al*., 1994; Hedborg *et al*., 1993; Sergeyeva *et al*., 2001; Sergeyeva *et al*., 1999; Ulbricht *et al*., 2002). The preparation of such membrane structures has been studied in terms of specific permeability and separation of templates/ligands. MIP membranes have become increasingly attractive due to their high affinity in separation technology including SPE (Solid Phase Extraction). One example of the use of MIP membranes for separation of target molecules from a mixture of several similarly structured compounds would be through *in-situ* polymerisation. However this practical application is difficult due to the small fluxes which are typically no larger than 10^{-3} mol m^{-2} h^{-1}. Alternatively, another approach would involve the grafting or casting of a MIP layer to the surface of a stable support material (Piletsky *et al*., 1999; Wang *et al*., 1997). Several approaches in the past have involved photografting of special photoreactive polymers (Wang *et al*., 1997) or polymerisation onto an entire surface of polypropylene microfiltration membranes (Dzgoev and Haupt, 1999). Recently, another design of MIP membranes has been studied which involved the surface modification of membranes by heterogeneous photografting. For such designs, a photoinitiator is required which would coat the polymer membranes, and after UV excitation produce radicals on the membrane polymer surface. The function of these radicals is to initiate the grafting copolymerisation of the functional monomers. In this way, a thin layer of a functional polymer would be created which would cover the entire surface area of the membrane (Sergeyeva *et al*., 2001).

The formation of MIP membranes has also being applied for conductimetric sensors. This work has been reported specifically for nucleic acid components, cholesterol and atrazine, based on free-standing and glass filter imprinted membranes (Piletsky *et al*., 1998). These MIP sensors demonstrated high stability, sensitivity and selectivity but poor processibility. The problem of the reproducibility and stability of thin membranes has been partially resolved by adding plasticizer into the polymer composition (Sergeyeva *et al*., 1999).

In view of the goal of this PhD thesis, where the development of an electrochemical sensor for herbicide analysis required the use of screen-printed technology, we attempted an alternative approach where biological sensing elements were

replaced with corresponding MIP specific for triazine herbicides. In this work a thin, flexible and mechanically stable MIP membrane has been produced using oligourethane acrylate (OUA) in a monomer mixture accordingly to protocol described elsewhere (Sergeyeva *et al*., 1999). MIP membranes were used as a base for screen-printed electrodes. A number of different initiators were tested to decrease the influence of oxygen on polymerisation reaction. A gold ink was screen-printed on top of the electrodes for both sides of the membranes in order to be used for conductimetric analysis of atrazine.

4.2 MATERIALS AND METHODS

4.2.1 General reagents

Di-trimethyl-silane and (tri)ethylene glycol dimethacrylate (TEDMA) was purchased from Fluka (Switzerland). Dimethylformamide (DMF), 1,1'-Azobis-(cyclohexanecarbonitrile), 2-Benzyl-2-(dimethylamino)-4'-morpholinobutylophenone 97%, Omega-omega-Dimethoxy-omega-phenylacetophenon 99% and methacrylic acid were purchased by Sigma-Aldrich. Toluene was purchased from Acros Organics (UK). Oligourethane acrylate (OUA) was kindly supplied by Pilot Factory of the Institute of Macromolecular Chemistry (Kiev, Ukraine). Atrazine crystals were dissolved in 1% methanol and further diluted with reverse-osmosis water to prepare a stock solution for further experiments. Gold conductors for screen-printing were purchased from Dupont (UK).

4.2.2 Molecular imprinted membranes

For the preparation of MIP membranes, methacrylic acid was chosen as the functional monomer, (tri)ethylene glycol dimethacrylate was chosen as the cross-linker and atrazine was the template. For an efficient interaction between the template and the polymer, a ratio of one atrazine to five methacrylic acids was chosen as described previously (Sergeyeva *et al*., 1999). For the mechanical stability of the membranes, OUA was added to the monomer mixture prior the polymerisation. A typical preparation of the MIP membrane was as follows: 20 mg of atrazine were mixed with 40 mg of methacrylic acid, 289 mg of TEDMA, 51 mg of OUA and 100 mg of solvent (DMF) containing 4 mg

of initiator. Three initiators were tested under the same conditions. The monomer mixture was poured between two quartz glasses held apart with Teflon film ranging from 60 to 180 μm. The two glasses were cleaned with a mixture of dichlorosilane/toluene with a ratio of 1:3 and held together with Swingline Steel presentation clips. To complete the polymerisation, the membranes were exposed to UV light (λ = 365 nm) for 30 minutes to 2 hours. The template molecules were extracted by washing with 50 mM HCl overnight. Next the membranes were washed with water and methanol (20 volumes each) and dried at room temperature. The corresponding blank membranes were prepared in the same way but in the absence of the template. This, resulted in the synthesis of thin, flexible membranes.

4.2.3 Screen-printed MIP membranes

Screen-printing required only two steps repeated twice to cover both sides of the MIP membrane with gold film. The electrode configuration was chosen according to the application of the sensor and the availability of the screens at Cranfield University. For the screen-printed technique, the specifications of the DEK 248 screen-printer had to be optimised from what that described before (see Chapter 3, Table 3.1) with the parameters shown in Table 4.1.

Table 4.1: Parameters set on DEK machine for screen-printing on MIP membranes.

Print parameters	Settings
Print gap	0.1 mm (depending on substrate)
Front limit	110 mm
Rear limit	230 mm

Gold ink was printed both sides and was separated into two unequal parts with an insulation layer. Thus, a sensor was created having a working area and connectors (Figure 4.2). The electrodes could not be dried at the usual temperature of 120 ^{0}C as the membranes tend to bend and therefore had to be left to dry under much milder conditions such as at room temperature overnight. This resulted in well cured, flat membranes ready for use. For the conductimetric method, the values were chosen from previous work (Sergeyeva *et al*., 1999).

The electrode was connected to the sensor through crocodile clips isolated from one side so that the sensor would respond as a closed circuit. The electrical conductivity was analysed in a 10 ml beaker having a 25 mM phosphate buffer and 35 mM NaCl. Conductivity was set to 60 mV. The output signal was recorded with a selective amplifier (Standard Research Systems/ Model SR830 DSP Lock-In Amplifier). The frequency was set to 100 kHz and a phase at + 0.00 degrees.

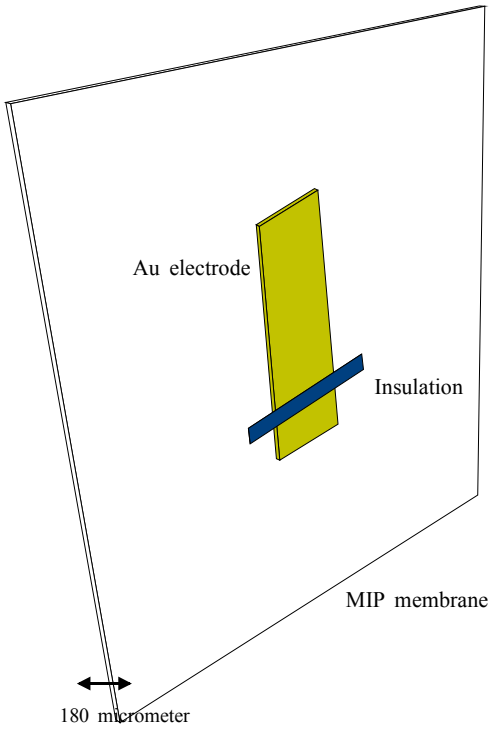


Figure 4.2: MIP membrane with Au screen-printed electrode.

4.3 RESULTS AND DISCUSSION

The synthesis of a highly specific MIP membrane requires the polymerisation step to be carried out under conditions where a strong monomer-template complex is retained during the polymerisation process. This is important as it is generally accepted that the strength of these complexes is reflected in the affinity and selectivity of the imprinted polymer. The membrane has to be able to be used in aquatic or buffer solution for the binding of atrazine. Therefore, the binding of the template to the polymer has to be strong in both media. Methacrylic acid was chosen as the functional monomer since it forms

strong complex with the atrazine molecule. For the synthesis of the membranes, copolymers with OUA were chosen for mechanical stability and reproducibility.

A set of flexible transparent membranes were synthesised through U.V. initiation and tested by conductimetric detection. Three initiators were tested each with the same polymerisation recipe. The 2-Benzyl-2-(dimethylamino)-4'-morpholinobutylophenone 97% produced a membrane which continued to polymerise under ambient light conditions. This was the only membrane that became purple in colour and this could be due to the amino group oxidation of the breakdown of the initiator trapped within the polymer. The 1,1'-Azobis-(cyclohexanecarbonitrile) and omega-omega-Dimethoxy-omega-phenylacetophenon 99% produced transparent membranes but of the two, the latter gave the more mechanical stable membrane. Membranes with thickness 60 μm were too thin to support screen-printing on and therefore broke during this process. The optimal distance of the quartz glasses was 180 μm for membranes able to be screen-printed on both sides together with insulation layer. The drawback of this was the slow kinetics of drying and equilibration during the analysis. Unfortunately, the membranes could not be washed with an organic solvent to facilitate drying as the layers of ink would be damaged.

As it was mentioned before the electrodes were connected to the conductimetric device through crocodiles clips isolated at one side in order to form an open circuit. The electrodes were dipped in a 25 mM phosphate buffer and the optimal pH value was adjusted to 7.5, as was previously reported by Sergeyeva *et al*. (1999). The response of the sensor was recorded against time. The signal increased gradually against time even when the sensor was left overnight. Atrazine additions of 60 ng/ml only limited the sensor response temporarily. The signal continued to increase after a period of 60 minutes and a stable plateau could not be reached (Figure 4.3). The reason for this could be the swelling of the membranes. Previous work has indicated a good atrazine response at pH 7.5 for membrane thickness of 60 to 120 μm. Swelling or shrinking of the membranes results in the loss of the ability of polymer sensor to generate stable signal. Diffusion of the template molecules would vary according to the thickness of the MIP membranes. Since the membrane continues to swell during the whole experiment, this also affected the diffusion of the template and made the reading of the signal non-reliable. The difference in behaviour of the free-standing membrane and the screen-printed membrane lies in the fact that the first was fully immersed into the solution and after 2 hours the swelling

process reached its maximum, which allowed continuous measurement starting from this point. In the second case only part of the membrane was exposed to the solution which made swelling process too slow.

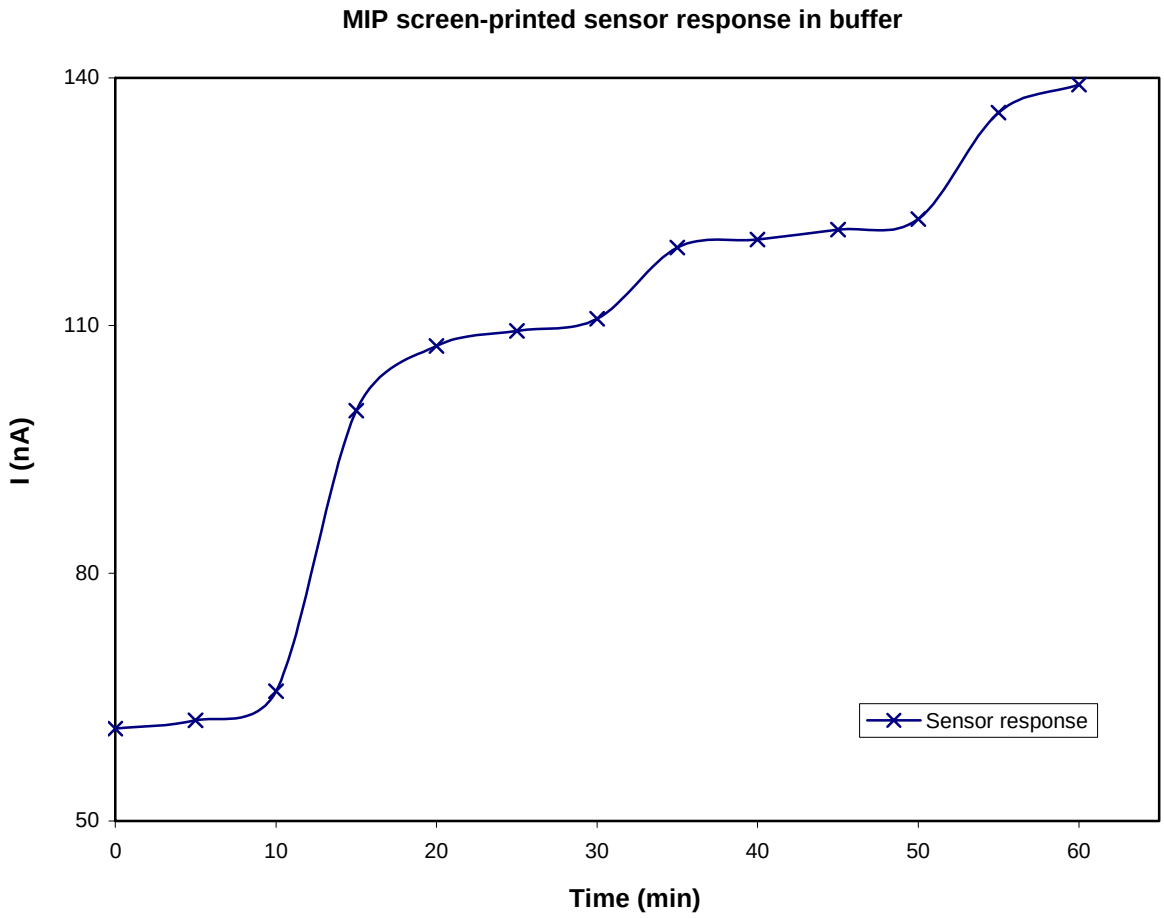


Figure 4.3: Sensor response for swelling of MIP membranes. Values on the y-axis represent current values (nA).

4.4 CONCLUSION

The production of MIP integration within screen-printing membranes discussed in this chapter has been feasible. The method chosen for the production of the MIP membranes for conductimetric measurements has been proven successful in the past. The approach described here, in which the transducer became fully integrated with membrane, is very interesting, and we managed to identify several problems which required solution before it would became feasible. Among these problems are: the slow swelling process which might require minimising the thickness of the membrane, and the need for

separation of the conductive parts from the main body of the membrane. For the future work we intend to work on the support which will provide better mechanical stability to the thin MIP membrane. This could be performed by either by doing the polymerisation step on filter paper or changing the configuration of the screen-printing connectors in a way that the membrane will be fully immersed in the solution.

CHAPTER 5. HYDROGEN PEROXIDE DETECTION

5.1 INTRODUCTION

As it was mentioned in the literature, hydrogen peroxide is considered a commonly detected analyte in the field of biosensors. The important areas of H_2O_2 application include industry (pharmaceutical, food, clinical), and environmental analyses. Its use as an antibacterial agent added to milk, and the environmental need to avoid halogenated substances for disinfection purposes, make H_2O_2 an important substance in the food and beverage industry. This raised an extensive demand for establishing the protocol for H_2O_2 detection depending on its application. A variety of analytical methods have being previously reported including spectrophotometry (Sellers, 1980) and chromatography (Qi and Baldwin, 1993). Additionally, it is one of the most important products or substrate of enzyme catalysed oxidation reactions. Besides being a product/substrate of enzymatic reaction, hydrogen peroxide is by itself an important analyte. It plays an important role in natural oxidation processes as it is found in air, solids and water.

Hydrogen peroxide is considered a powerful oxidant; more powerful than chlorine, chlorine dioxide, and potassium permanganate. Through catalysis it could be converted to hydroxyl radicals through the following mentioned oxidation potential (Table 5.1).

Table 5.1: Reactivity**.**

Oxidant	Oxidation potential (V)
Fluorine	3.0
Hydroxyl radical	2.8
Ozone	2.1
Hydrogen Peroxide	1.8
Potassium permanganate	1.7
Chlorine dioxide	1.5
Chlorine	1.4

Despite being a powerful oxidant, it is generally quite safe as a natural metabolite of many organisms as they decompose it producing oxygen and water. Furthermore, H_2O_2 is considered a natural purification element in the environment as it can be produced by the interaction of sunlight with water. As a consequence, it does not have the problems that other oxidants have, such as release of a toxic gaseous product, ions or harmful chemical residues which persist in the environment.

The direct electrochemical detection of hydrogen peroxide via oxidation or reduction reactions has been attractive enough. However as it has been mentioned, this process would require large overpotentials which automatically means interferences from all oxidisable substances that would be present in the sample. The use of mediators either immobilised or deposited on the electrode surface has found a widespread application in order to eliminate the above mentioned phenomena. From section 2.3 equations 2.3, 2.4 and more specifically from section 2.3.3 equation 2.10 a general form arises for mediated detection of hydrogen peroxide.

$$H_2O_2 + \text{oxidised mediator} \rightarrow O_2 + 2\,H^+ + \text{reduced mediator} \quad \textbf{(5.1)}$$

$$\text{reduced mediator} \rightarrow \text{oxidised mediator} + ne^- \quad \textbf{(5.2)}$$

Reaction 4.1 demonstrates an electron transfer from H_2O_2 to the mediator making it reduced on the electrode surface. Following this, the reduced mediator is itself re-oxidised electrochemically giving a rising current as a result of reaction 4.2. Examples of mediators have been mentioned previously. The ones used for this work include hexacyanoferrate (Amine *et al*., 1993), quinones (Vidal *et al*., 2004) and ferrocyanide (Collyer *et al*., 2003; Liu *et al*., 1996). Addition of soluble mediators would further require the storage, transport and handling of one more reagent and therefore its immobilisation would be essential for the development of a suitable portable device.

As reported in section 2.3.5, metallised electrodes help to minimise overpotential and could be compared with soluble mediators. Detailed discussion of mediated electrodes could be found in Gorton *et al*. (1991). Electrode surfaces could be modified by either using entirely noble metals such as gold, platinum, palladium, ruthenium and rhodium or by incorporating metal particles in the carbon. A cost effective development of different materials and production methods have been previously reported. Of all of

them, rhodium modified carbon was found to be a catalytic material with good properties for the oxidation of hydrogen peroxide. As a result of this and its ease of production technique, it was chosen as the one of the modes of screen-printed electrodes in this project along with platinum electrodes provided from Krejci engineering and the carbon/polymer electrode as a novel part of the work.

5.2 MATERIALS AND METHODS

5.2.1 Reagents

Hydrogen peroxide (30% w/v), potassium chloride (KCl), horseradish peroxidase (Type II), potassium ferrocyanide ($K_4Fe(CN)_6$.3 H_2O), and hydroquinone (1,4-Benzenediol 99%) were from Sigma. Hydrogen peroxide was stored at 4^0C during measurements. This stock solution was assumed to be 8.88 M and a series of dilutions were prepared daily, having different molarities, using RO water to minimise any effect from catalytic decay. To make a buffer of pH 7.4 the following reagents were used, di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4) and sodium dihydrogen orthophosphate anhydrous (NaH_2PO_4); these were purchased from BDH Laboratory Supplies, Merk Eurolab.

5.2.2 Safety considerations

Hydrogen peroxide is a powerful oxidant. The mixture of aqueous peroxides along with organic compounds could create an explosion. It is better to avoid skin contact but in the case of an accident it is necessary to rinse with water and seek medical advice. Gloves were worn all time either for handling peroxides or hydroquinone. Consideration of safety information for hydroquinone revealed that it is harmful if swallowed; there is limited evidence of a carcinogenic effect. Furthermore, it could cause sensitisation by skin contact and has serious effect to eyes. It is very toxic to aquatic organisms, light sensitive and air sensitive. In cases of skin contact it is advisable to remove all clothing and wash thoroughly with water. In case of breathing problems and limited fresh air, it is better to give artificial respiration and if the affected person is still suffering, give oxygen. In all cases medical advice should be sought (Fisher Scientific/Acros Organics).

5.2.3 Electrochemical analysis

For electrochemical measurements, all apparatus and conditions have been described in section 3.2.2 (Chapter 3). In early stage measurements, electrodes were immersed in a 10 ml phosphate buffer solution containing a 0.1 M potassium chloride (KCl), using a 10 ml beaker. All three types of screen-printed electrodes (working, reference and counter) were covered from the buffer solution (below surface). For chronoamperometric measurements, H_2O_2 was not present in the stirred solution, but it was added once a current plateau was established and that was typically 1 to 3 minutes. In case of voltammetric measurements, the solution was not stirred and H_2O_2 was present in the buffer from the start of the measurements. In this case one minute of stirring was performed prior to start the analysis.

5.2.3.1 Direct and indirect H_2O_2 measurements

Direct electrochemistry of H_2O_2 was carried out in a solution containing 40 mM phosphate buffer and 0.1 M KCl on all types of electrodes. Amperometric scans were set at a certain potential, depending on the type of the electrode and were run for 400 seconds.

Indirect measurements were performed using both enzyme and mediator for all types of electrodes including metallised ones. The measured sample contained 300 µl of 0.05 mg/ml HRP, and 50 µl of 100 mM hydroquinone (HQ) solution prepared in 40 mM phosphate buffer pH 7.0 and 0.1 M KCl. For the physical adsorption 50 µl of 0.5 mg/ml solution of HRP was left to dry for an hour on the working electrode surface. Concentrations varying from 1 % - 10^{-4} % of hydrogen peroxide were prepared from a 30 % stock solution. Additions of 50 µl of H_2O_2 were made for each measurement after which each electrode was replaced with a new one.

Slightly different potentials were chosen for each type of electrode according to cyclic voltammetry data. Response time however remained the same (100 seconds). Electro-reduction started at + 0.3 V for carbon and at -0.4 V for rhodonised electrodes. A comparative study was prepared between these two potentials. Polymer/carbon electrodes

gave similar voltammogram as carbon. Cyclic voltammograms of carbon (Figure 5.1), rhodonised (Figure 5.2), and platinum electrodes (Figure 5.3) are shown below.

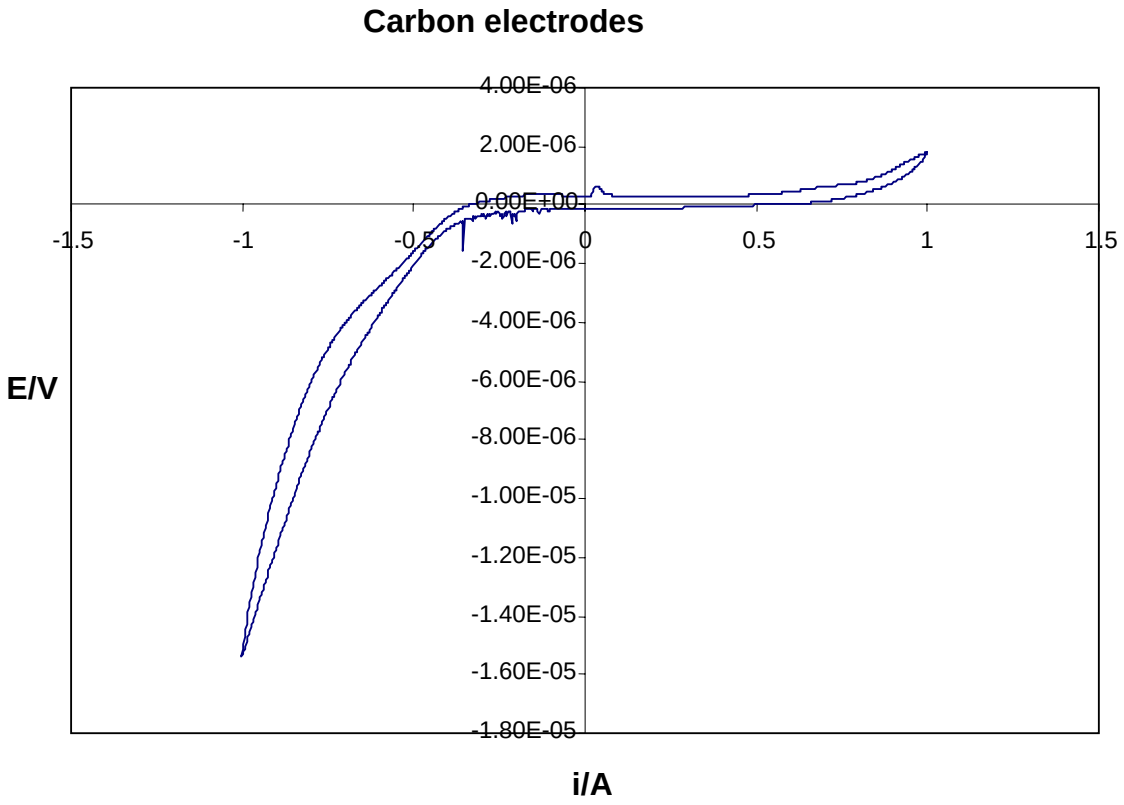


Figure 5.1: Cyclic voltammetry of carbon electrodes.

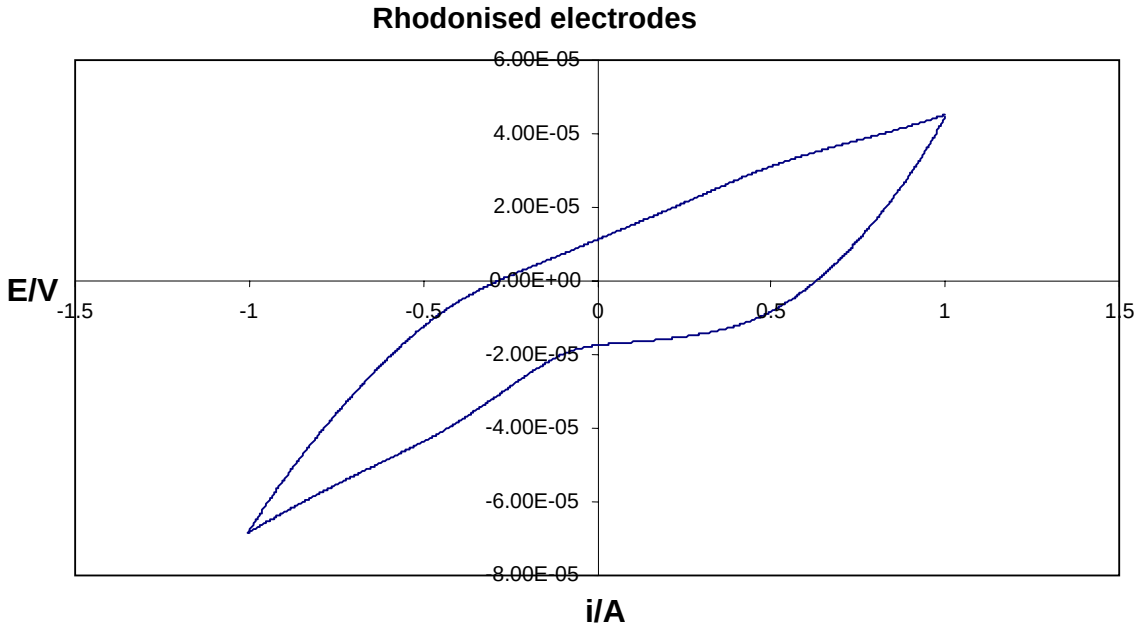


Figure 5.2: Cyclic voltammetry of rhodonised electrodes.

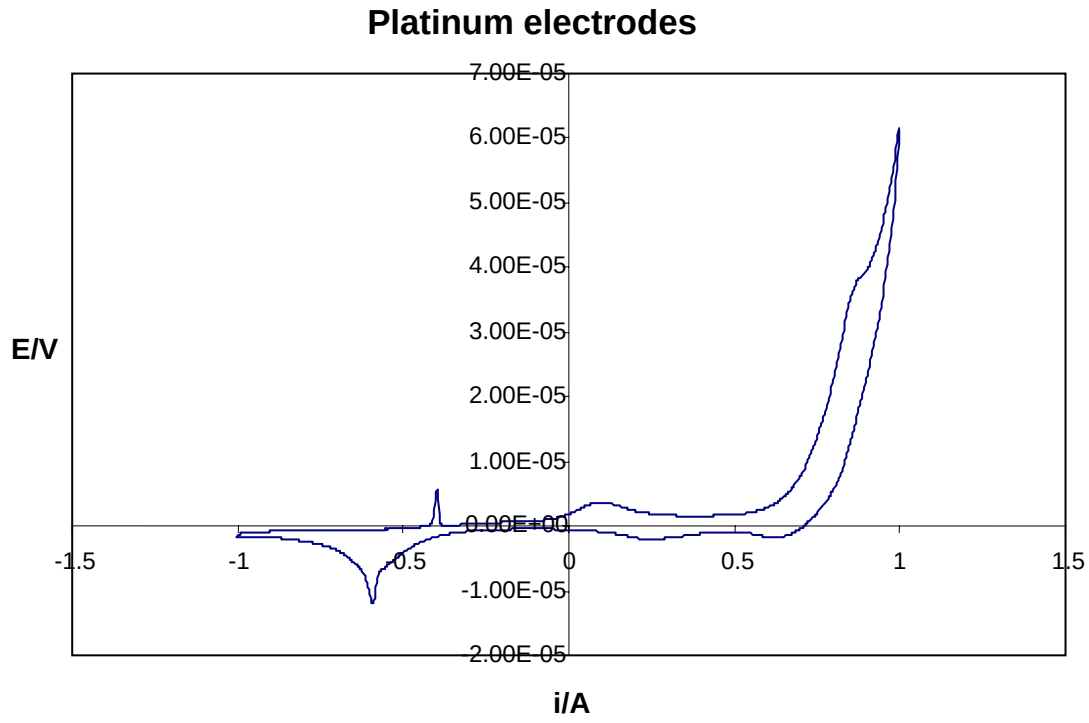


Figure 5.3: Cyclic voltammetry of platinum electrodes.

5.2.3.2 Flow injection analysis for H_2O_2 measurements

Flow injection analysis (FIA) has been used for hydrogen peroxide measurements using carbon electrodes. Flow injection has been selected, since further integration of the sensor for herbicide measurement will require the use of flow cells (microflow). That was necessary for reproducing industrial operating conditions applied in a laboratory environment. Thus, dilution in a flowing buffer would be essential with the use of a sampler, pump and flow cell arrangement.

FIA is a powerful technique which allows the detection of a solution with a high degree of automation and relatively inexpensive equipment. Experiments were made using a running solution of PBS (pH 7.4) and 1 mM hydroquinone on a carbon electrode with HRP physically adsorbed on the surface. Injections were made of 20 μl hydrogen peroxide (0.025%-0.001%) dissolved in PBS/HQ solution. The results were used to determine the detection limit for hydrogen peroxide in FIA. After each injection the sample loop was washed with an additional volume of buffer to wash the internal walls. For these measurements a 3 position, 7-port valve injector was used (Valve V-7) (Figure

5.4). A 20 µl injector loop was connected to the valve and the whole system was operated with the aid of a Hewlett Packard/Agilent pump series 1050 (HP1050) connected to the flow cell that enclosed the screen-printed electrodes.

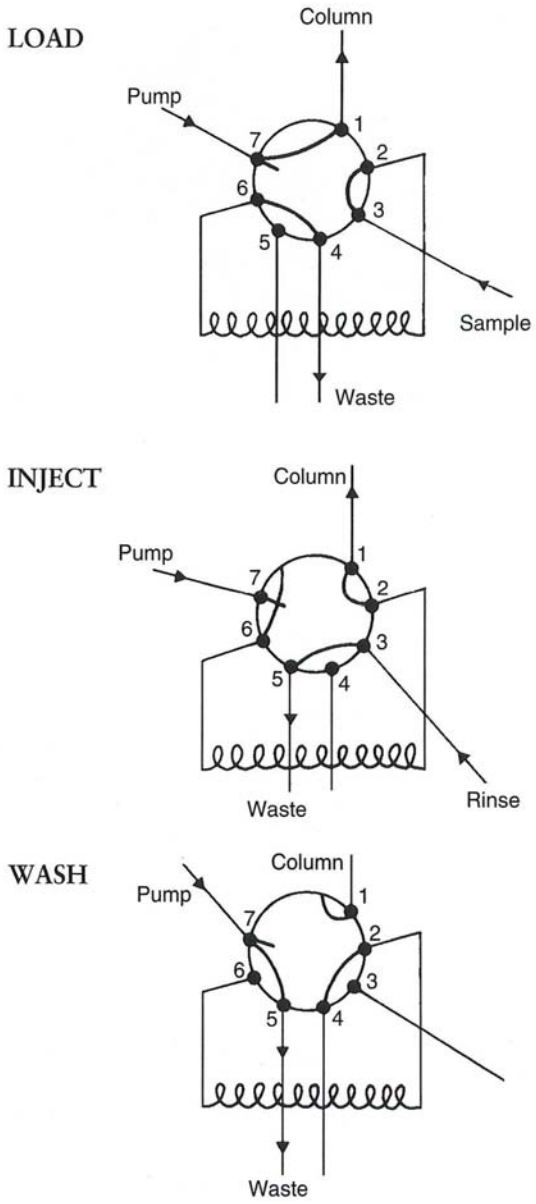


Figure 5.4: Valve V7 for flow injection analysis, with regulated injector loop according to the volume of sample and a 3 way system consisting of Load, Injection and Wash paths (Pharmacia).

The flow rate of the HP1050 pump could be adjusted according to application. It has control panels which makes it easier to diagnose system problems. The quaternary pump (79852A) is part of a system that includes an autosampler, solvent module, Helium degasser, HP thermostat column compartment, and either a diode array detector, wavelength detector or fluorescent detector. This whole system makes up the HPLC model 1050. The important parameters of the instrument operation are shown on Table 5.2. During measurements the flow was set to 0.5ml/min at the pressure to 1 bar. Conditions remained the same throughout the analysis carried out at several hydrogen peroxide concentrations.

Table 5.2: Hewlett Packard/Agilent pump series 1050 (HP1050) parameters.

Flow range	Setpoints from 0.001 to 9.999 ml/min in 0.001 ml/min increments
Flow precision	< 0.3% RSD (typically < 0.15 %), based on retention time at 0.5 ml/min and 2.5 ml/min
Pressure	Operating range from 0-400 bar up to 5 ml/min; from 0-200 bar up to 10 ml/min
Compressibility compensation	User-selectable, based on mobile phase compressibility
Recommended pH range	2.3 to 12.5
Gradient formation	Low pressure quaternary mixing/gradient capability using proprietary high-speed proportioning valve
Composition Range	0 to 100% in 0.1% increments from four independent channels
Methods	Battery-backed storage of up to 10 methods
Analog Output	For pressure monitoring, 2 mV/bar
Flow range	Setpoints from 0.001 to 9.999 ml/min in 0.001 ml/min increments

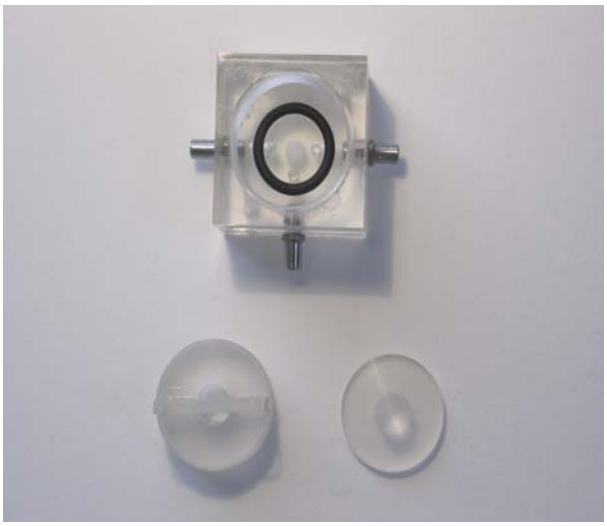


Figure 5.5: Photograph of the flow cell for the Cranfield SPCE.

For the design and construction of the flow cell, the specification of the screen printed electrodes prepared at Cranfield was used. The electrodes used for this analysis were the ones prepared at Cranfield University. The output of the final cell is shown in Figure 5.5. The cell was engineered and provided by Krejci engineering which was also a partner in the EU project. The microflow cell was tested for the analysis of hydrogen peroxide with the aid of horseradish peroxidase and hydroquinone. Two cells were received, one with a light source and one without. Since no further materials were received by the EU partners, only the cell without the light source was tested. The cell comes in two halves; they are sealed together and there is an 'O' ring seal which sits in a groove and is slightly deepened in the surface. Thus, the sensor is positioned in place and the 'O' ring creates a seal when the cap is tightened.

5.2.4 Polymer preparation for enzyme assay

The polymer/carbon electrodes mentioned in chapter 3 were further used in an enzyme assay. With the aid of a hole punch 12 disks with 6 mm diameter were cut from working surfaces of electrodes and placed in 2 ml eppendorf tubes. Five different concentrations of HRP with concentration varying from 0.5, 1, 1.5, 2 mg/ml were prepared in 50 mM phosphate buffer, pH 8.0 and 0.1% Tween and added to the tubes which were placed in the fridge for one half day (Figure 5.6). Protein immobilisation was analysed by ABTS method measuring the peroxidase activity of enzymes immobilised on the electrode (Piletska *et al*., 2000). Control experiments were run with electrode in the

absence of enzyme. The signal of these experiments was used as background and subtracted.

After HRP adsorption each disc was washed 5 times with 50 mM Na-phosphate buffer, pH 7.0 containing 0.1% Tween 20. ABTS (6 mg) was dissolved in 10 ml Na-citrate buffer (0.1 M, pH 6.0) and mixed with 3 µl 30 % H_2O_2. The discs were incubated in 100 µl of ABTS reagent for 10 min at room temperature. Next, the discs were removed and the optical density of the solution was measured at 450 nm. All analyses were repeated in triplicate. The amount of adsorbed HRP per m^2 of membrane surface was calculated from the corresponding calibration curve.

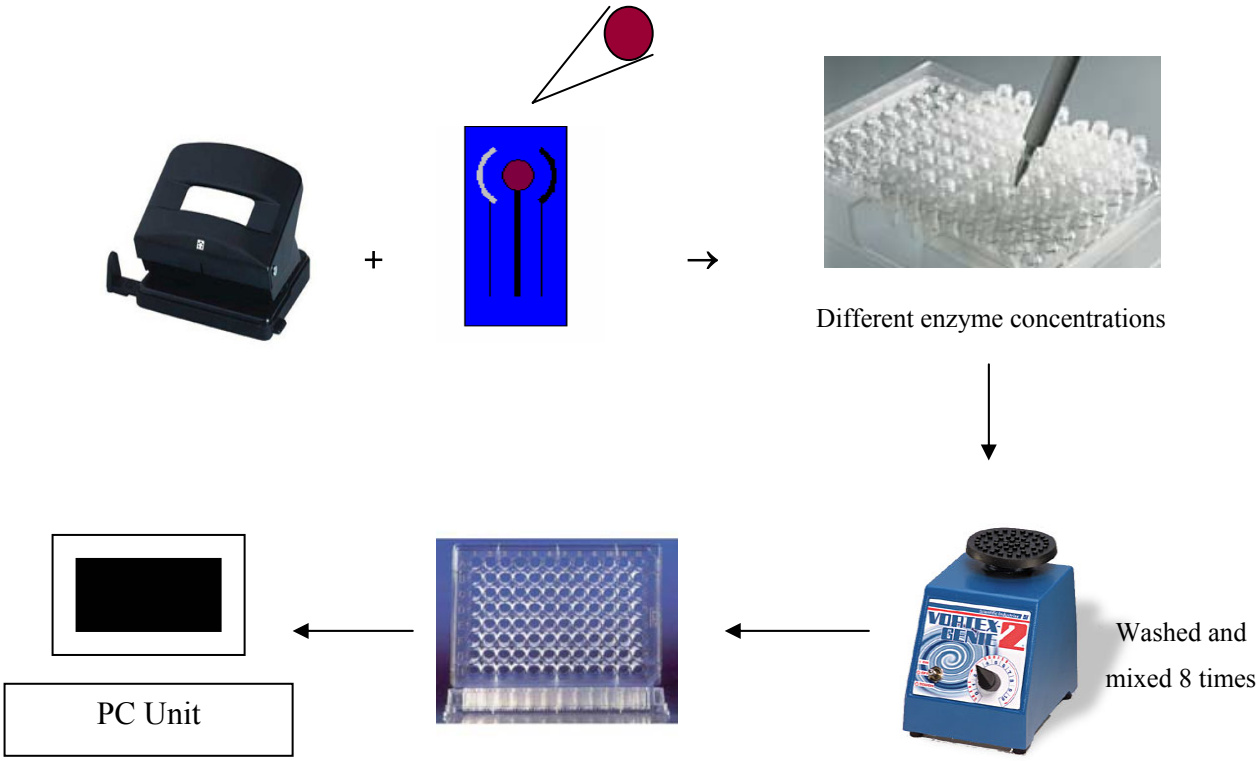


Figure 5.6: Protein Assay with optical detection.

5.3 RESULTS AND DISCUSSION

5.3.1 Use of mediators

SPCEs were further used with cyclic voltammetry for hydroquinone and duroquinone. CV allows the rapid location of the potential at which the analyte undergoes reduction and oxidation. CV of hydroquinone was reasonably simple (Figure 5.7). Duroquinone (Figure 5.8) was not soluble in phosphate buffer and needed the aid of an organic solvent. Its CV at a scan rate of 50 mV showed the presence of two oxidation peaks which could be explained through the chemistry of quinone compounds.

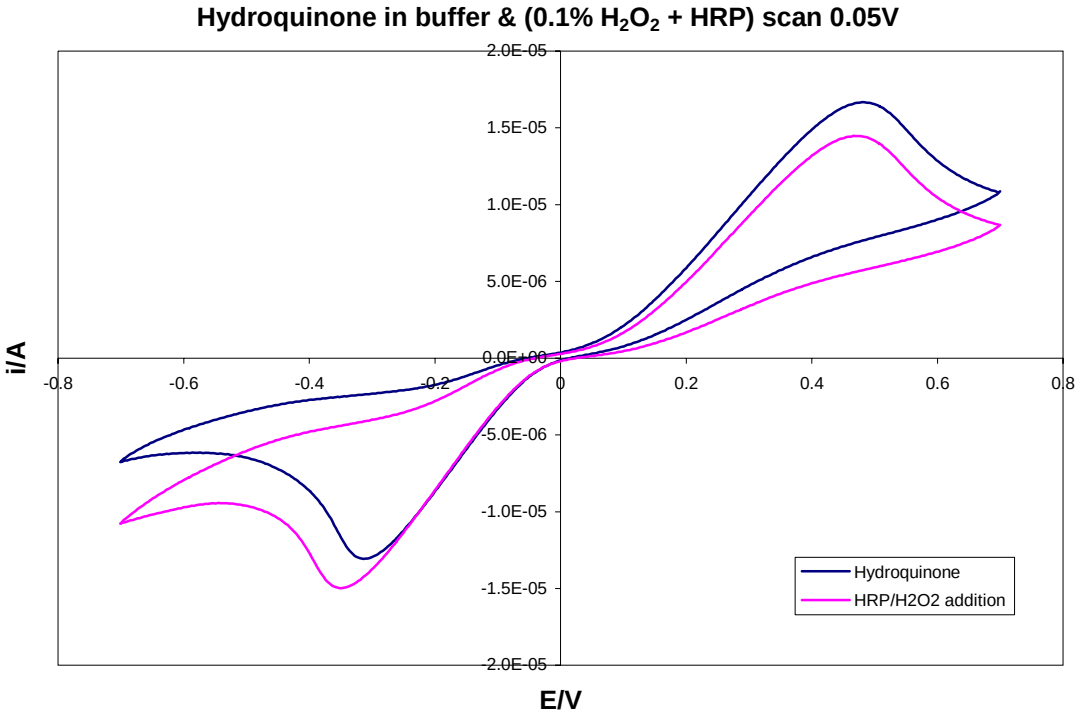


Figure 5.7: Cyclic voltammetry of hydroquinone (HQ, 100mM) at a scan rate of 50 mV in the presence of HRP (0.5mg/ml) and H_2O_2 (0.1%) on carbon electrodes.

Duroquinone measured at a high scan rate produces a by-product and therefore shows two oxidation peaks instead of one. At a low scan rate, these peaks overlap and appear as one. Therefore, CV for duroquinone measured at a low scan rate has the oxidation potential characteristics useful for amperometric measurements. Since it was necessary to use an organic solvent with duroquinone, to avoid it we chose hydroquinone for further

applications. Hydroquinone is used in the following experiments as a mediator in HRP immobilised electrodes having H_2O_2 as the substrate due to the reasonably straightforward electrochemistry of this compound. A reduction potential of -300 mV was used.

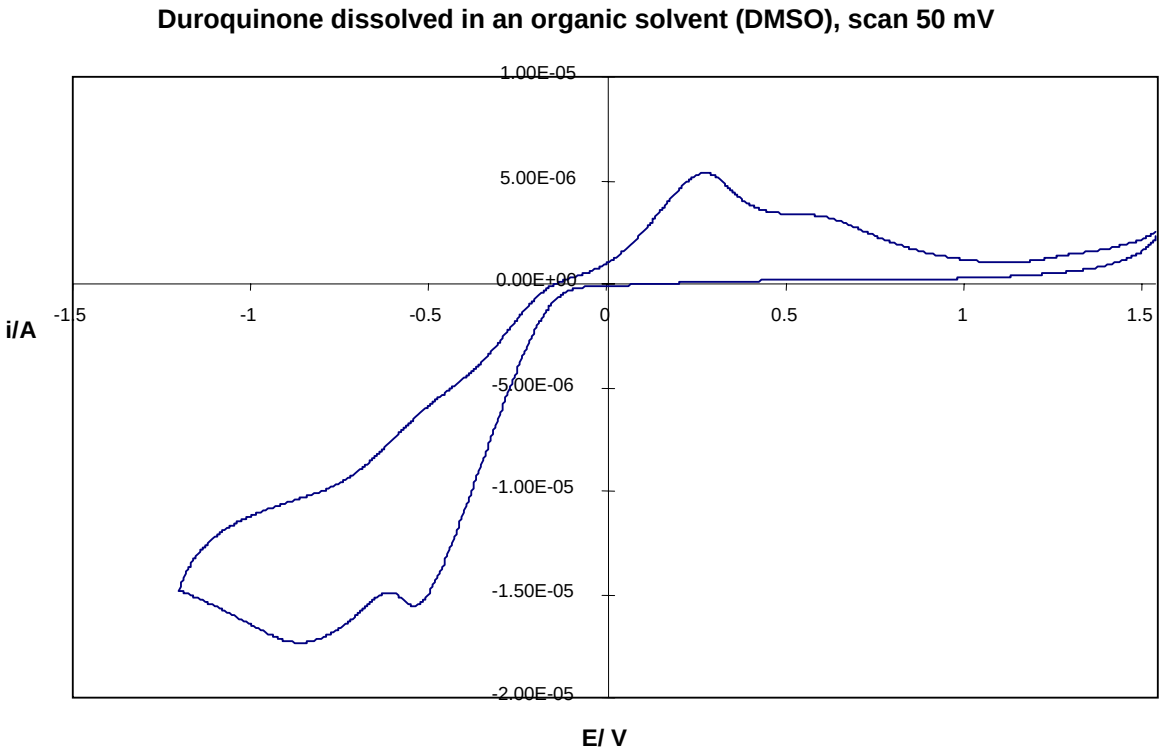


Figure 5.8: Cyclic voltammetry of duroquinone (DQ) dissolved in an organic solvent (DMSO-dimethyl sulfoxide) at a scan rate of 50 mV, measured on carbon electrodes.

Figure 5.9 shows a schematic representation of electron transfer that involves hydroquinone as the mediator, horseradish peroxidase as the enzyme and hydrogen peroxide as the substrate (Davis *et al*., 1995).

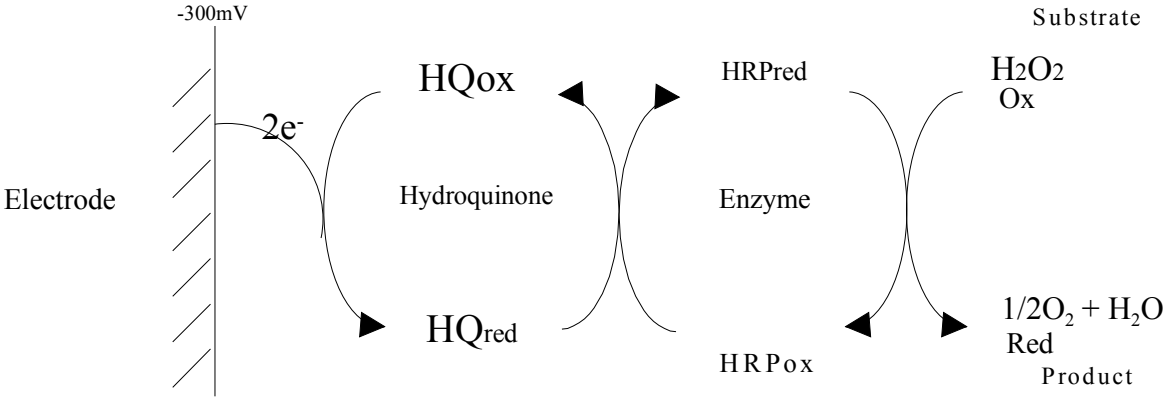


Figure 5.9: Electron transfer involving hydroquinone as the mediator at the reduction potential of -300 mV.

5.3.2 Enzyme integration with electrode

As discussed in section 2.2, there are several peroxidases which could be used in detection of hydrogen peroxide. Some of the most common ones are microperoxidase (Yabuki *et al*., (1999); Padeste *et al*. (2000); Huang *et al*., (2003)), catalase (Campanella *et al*., (2001)), and horseradish peroxidase (Ferri *et al*., 1998).

Horseradish peroxidase has been used frequently in ELISA systems and in biosensors for the detection of hydrogen peroxide. There is however small drawback, since H_2O_2 concentrations higher than 10^{-3} mol per l inhibit the enzyme activity (Bickerstaff, 1997). This should not be a problem as long as the maximum possible concentration is known for any particular application of the sensor.

There are many approaches which link the enzyme directly to the electrode for direct and mediated electron transfer (Cardosi and Birch, 1993, Cardosi and Turner, 1991). The enzyme can also be in free solution, it can be physically adsorbed on the working surface or covalently immobilised to the electrode surface. The preferred approach for enzyme immobilisation would be covalent immobilisation or entrapment in a gel. Various other possibilities include glutaraldehyde cross-linking, strong non-covalent bonding with biotin/avidin, or even sol-gel immobilisation (as discussed in literature review section). Cosgrove *et al*. (1988) and Liu *et al*. (1996) have shown that

immobilisation with glutaraldehyde and bovine serum albumin can be useful for stabilising the enzyme on the surface of gold electrodes. Unfortunately glutaraldehyde inhibits enzyme activity and decreases the sensitivity of the measurements. This approach, similar to many other techniques such as sol gel immobilisation used by Li *et al*. (1996) and Wang *et al*. (2000) appeared to be unsuitable for a mass production of biosensors.

In the present work we tested two approaches – physical adsorption and covalent immobilisation for integration of horseradish peroxidase with the electrode surface. Horseradish peroxidase was tested in this work in solution or by coating the working surface of the electrode and running a cyclic voltammetry scan between -1 V and +1 V in phosphate buffer pH 7.1.

Polymers are broadly used as immobilisation matrices in the development of biosensors. One attraction in such applications is that the polymers could be controlled in terms of the film thickness, porosity, quantity and quality of the functional groups and therefore used for control of the magnitude and rate of sensor response. We have developed a new thioacetale-based polymer capable of covalent binding to primary amines, e.g. proteins and nucleic acids (Piletska *et al*., 2000).

The reactive polymer is shown in Figure 5.10. This reactive hemithioacetale which is formed by allyl mercaptan and dialdehyde, is able to bind to primary amino groups. The formation of the isoindole complex could help realise the binding of the polymer to protein molecules. The kinetics of the reaction is slower than with low-weight organic amines. This is due to the formation reaction used to form isoindole which is partially reversible and dependent on the binding constant which is proportional to the number of interactions between the polymer and the analyte. Therefore, the binding capacity of the polymer is dependant on the number of amines available which is a function of the protein's structure and its amino acid composition. In this work thioacetale based polymer were used as an immobilisation matrix in developing enzyme sensors. The polymer was integrated with screen printed electrodes by mixing it with carbon paste at a ratio of 1 to 6. Thioacetale containing screen printed electrodes were coated with HRP by immersing them in a phosphate buffer containing enzyme. HRP was covalently attached to the polymer/carbon surface by binding of the thioacetale to the amino group of the protein. Physical adsorption of the carbon surface was not so efficient but still the difference

between the two electrodes was good enough. The efficiency of enzyme immobilisation was tested by colorimetric assay (Figure 5.11). Control experiments were run with substrates, but in the absence of the enzyme. The signal of the controlled experiments was used as background and was subtracted from the values obtained for peroxidase activity of electrodes on to which enzymes have been immobilised. When the working surface of the electrodes was removed, the optical density of the solution was measured at 450 nm and compared with solutions containing known concentrations of enzyme (calibrated samples). The immobilisation density for enzymes was 30-100 ng/cm^3. Polymer modified electrodes showed reproducibility similar to non-modified electrodes (7% RSD). Screen-printed polymeric electrodes offer a handy tool for the production of biosensors, which are easy to handle, inexpensive, highly reproducible and disposable.

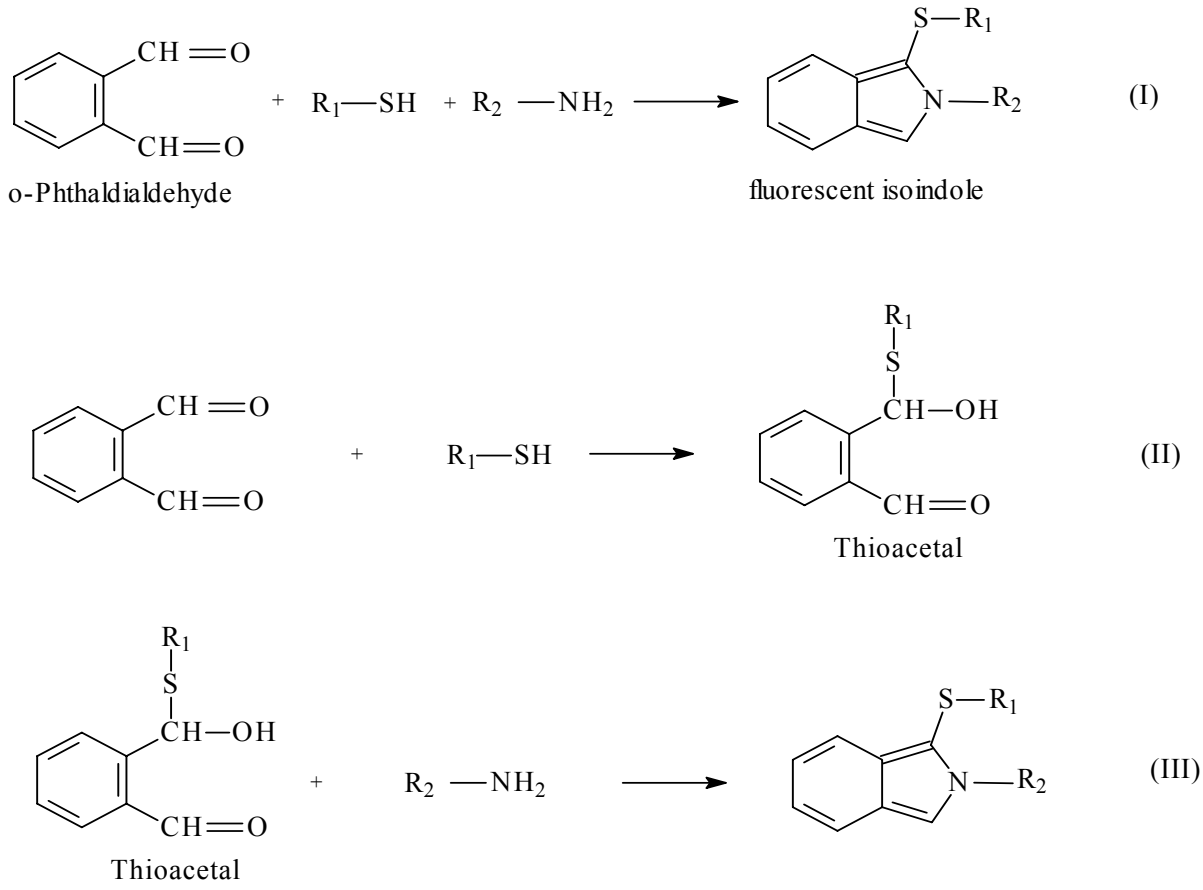


Figure 5.10: Primary amine (I), hemithioacetale formation (II), isoindole complex formation between hemithioacetale and primary amine (III). R_1-SH- polymerisable mercaptan, and R_2-NH_2- primary amine.

The adsorption of HRP for the carbon/polymer electrodes was found to be almost five times higher than that for the unmodified (carbon) electrodes. This result justified the use of these types of electrodes for further measurements.

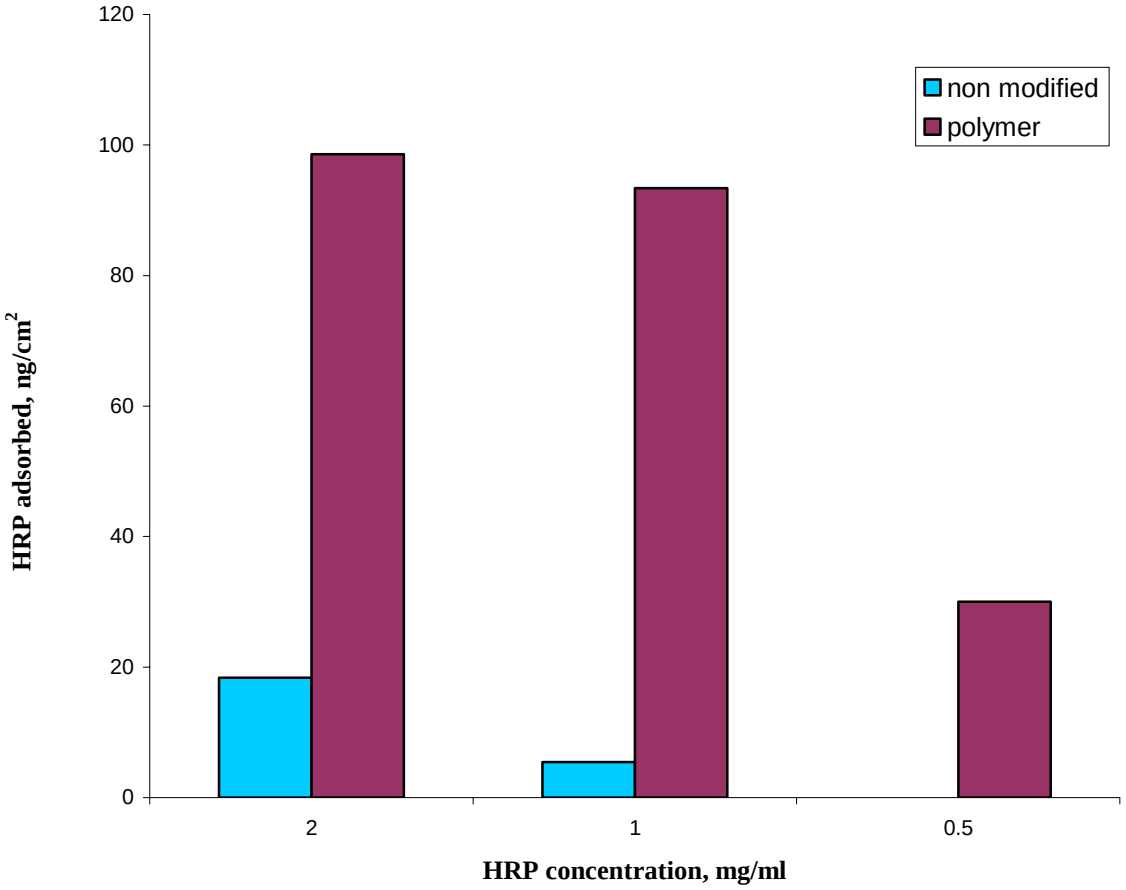


Figure 5.11: Results of a protein assay for a new reactive polymer added in carbon paste for enhancement of the paste and more sensitive detection of hydrogen peroxide.

5.3.3 Direct amperometric detection of H_2O_2

Direct measurements of H_2O_2 were performed in all types of electrodes. The potential for each type of electrode was taken from literature (for rhodonised, platinised and electrodes provided from Krejci engineering) or by step amperometry (carbon electrodes), and cyclic voltammetry (electrodes employing hydroquinone). Using all available types of electrodes, amperometric detection of hydrogen peroxide was performed with (indirect measurement) and without (direct measurement) enzyme. The

relevant oxidation potential was used for each type of electrode. At each potential, successive additions of H_2O_2 were made and the amperometric signal obtained (Figure 5.12) was measured and calibrated. Amperometric measurements were run for 300 seconds monitoring sensor response to the concentrations of H_2O_2 ranging from 1 mM to 1 μM. When going from higher to lower concentrations the signal was decreased and eventually disappeared, mean establishing the lower limit of detection with each type of electrode. The measured difference between the background current observed (100 seconds) and the peak height (300 seconds) was used for calibrating the data. The plot obtained represents time in the x axis, versus current in the y axis.

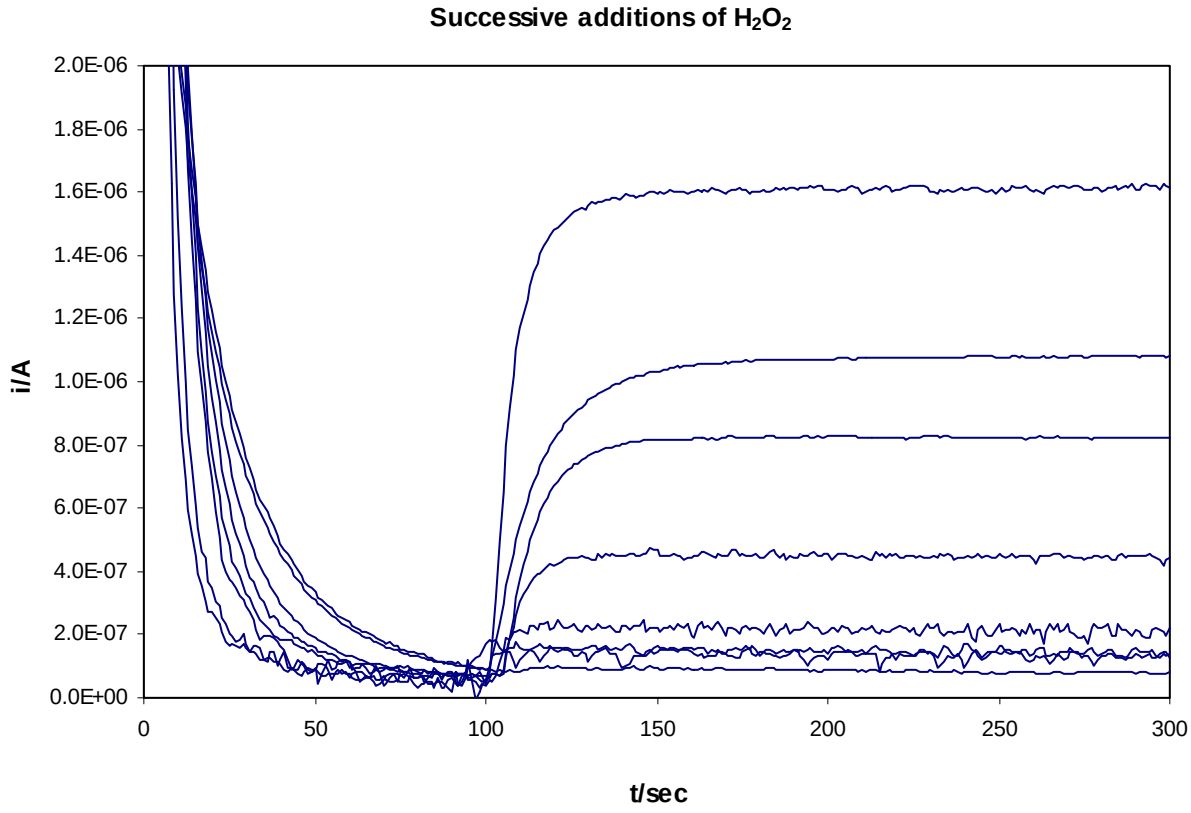


Figure 5.12: Successive additions of hydrogen peroxide in chronoamperometry.

Having plotted the data, measurements of the standard deviation and the slope of the calibration line gave the limit of detection for each type of electrode. For plain carbon electrodes the optimum potential was 600 mV (Figure 5.13). The potential range was swept between 0 and 1 V and in every potential step the response of hydrogen peroxide was recorded. The potential step that provided the highest response, after subtracting the

blank, was chosen for further experiments. The calibration curve for carbon electrodes at a potential of 600 mV show that they were able to detect H_2O_2 from 1.48 mM to 0.015 mM level (Figure 5.14).

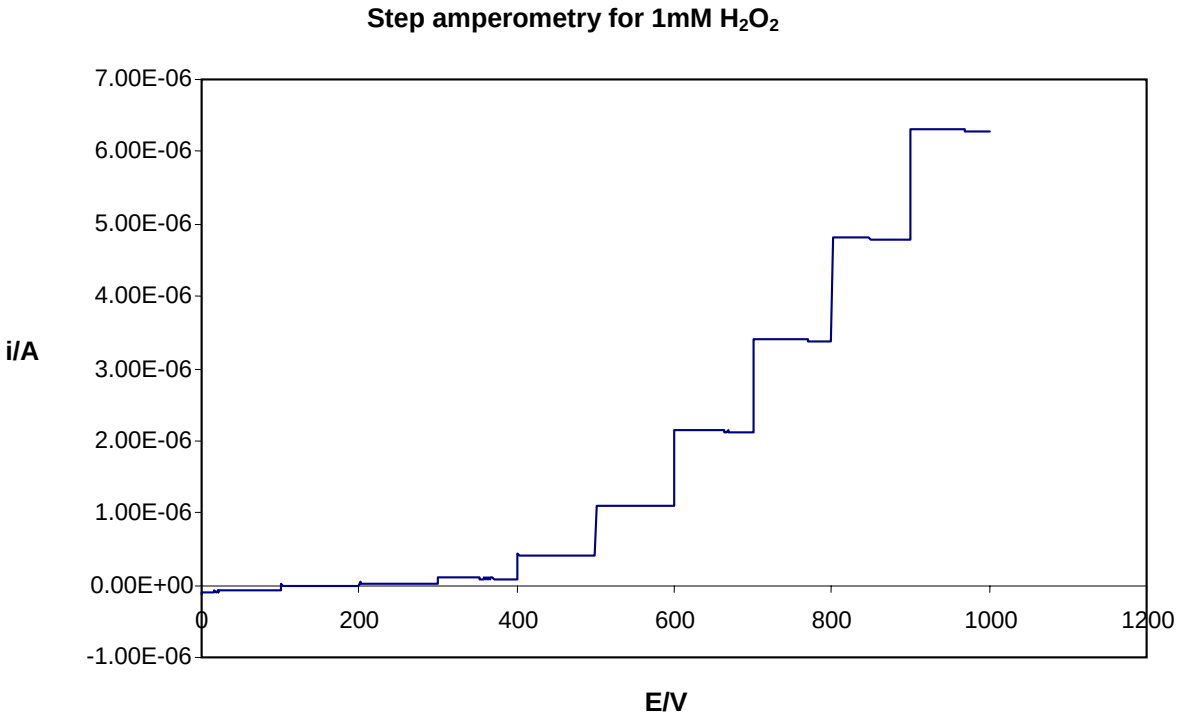


Figure 5.13: Step amperometry of carbon electrodes for 1 mM H_2O_2 at a potential of 0-1V.

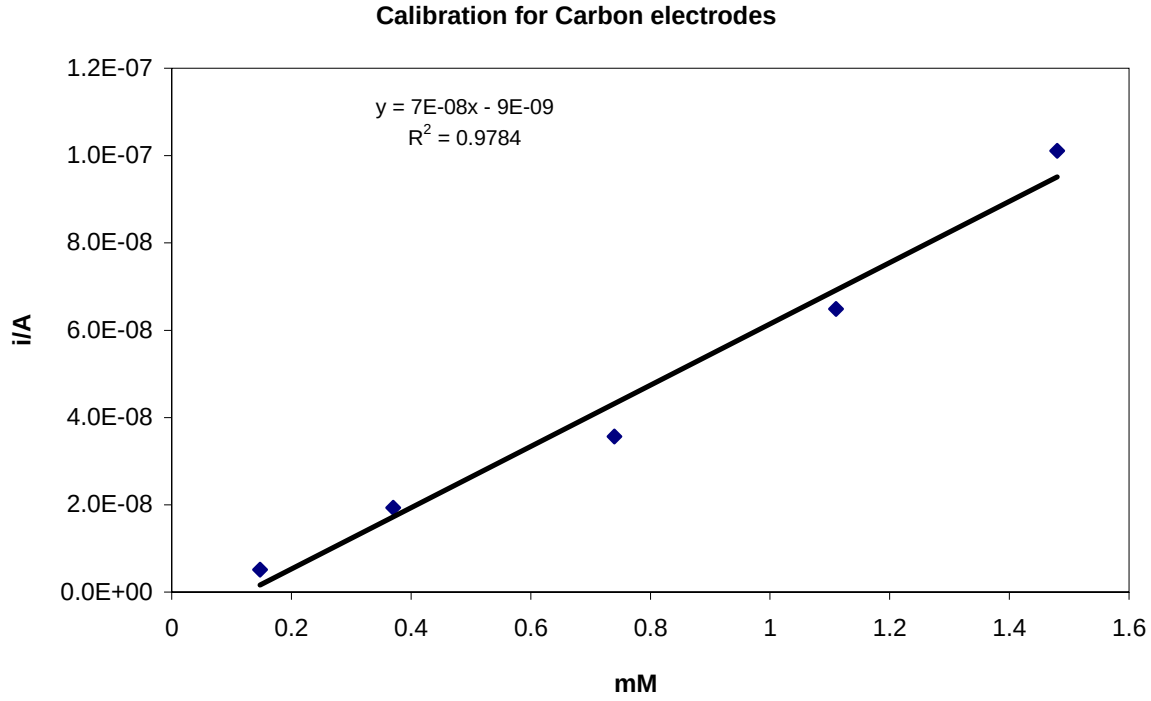


Figure 5.14: Hydrogen peroxide detection at carbon electrodes at a potential of 600 mV.

Since the potential is quite high and the detection is not sensitive enough, the rest of the carbon electrodes were used with enzyme either in free solution or physically adsorbed on the electrode surface. Platinum (Figure 5.15) and rhodonised (Figure 5.16) electrodes gave more sensitive results in direct measurements of hydrogen peroxide. At potential of 550 mV, platinum electrodes gave a detection limit of 1.86 μM compared to the 0.49 μM of the rhodonised electrodes at the potential of 300 mV. At a potential of 600 mV carbon electrodes with acetate cellulose (L4) (Figure 5.17) gave a limit of detection around 0.134 mM which again is as high as the carbon electrodes produced at Cranfield. Finally L5 type of electrodes at the same potential gave an even less sensitive detection limit of 7.23 mM (Figure 5.18).

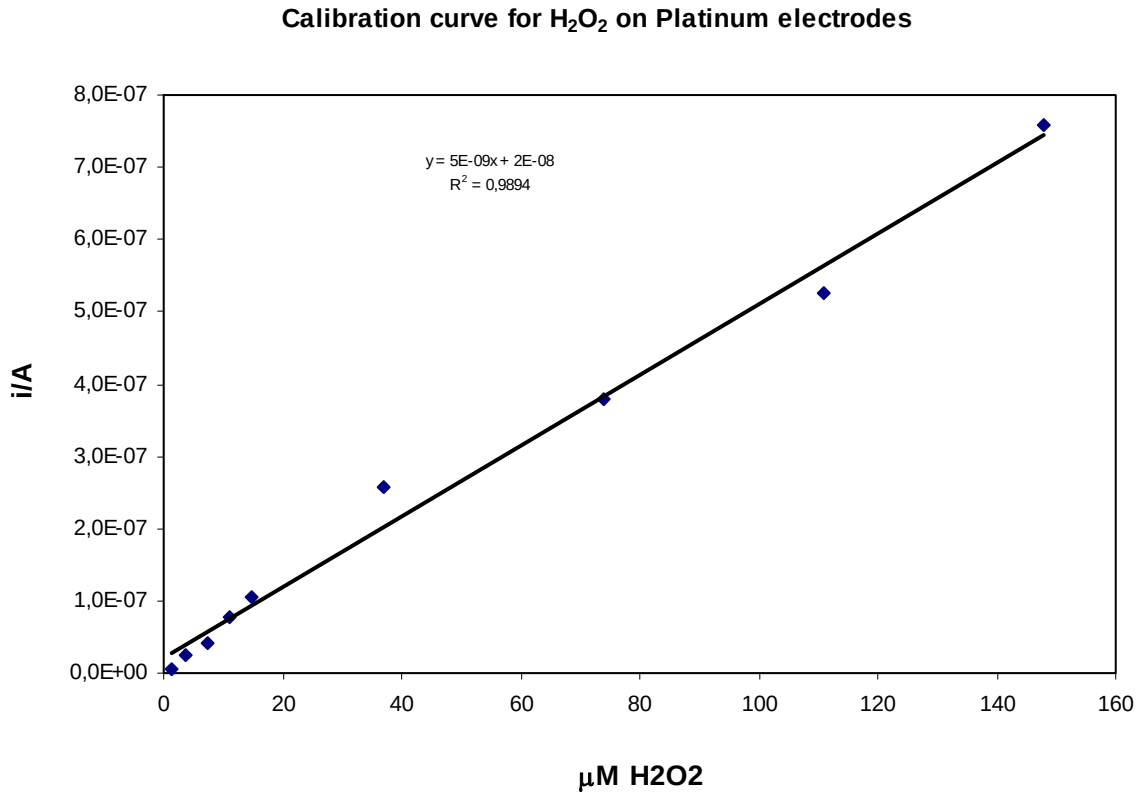


Figure 5.15: Detection of hydrogen peroxide on platinum electrodes (AC1.W2.RS) at a potential of 550 mV.

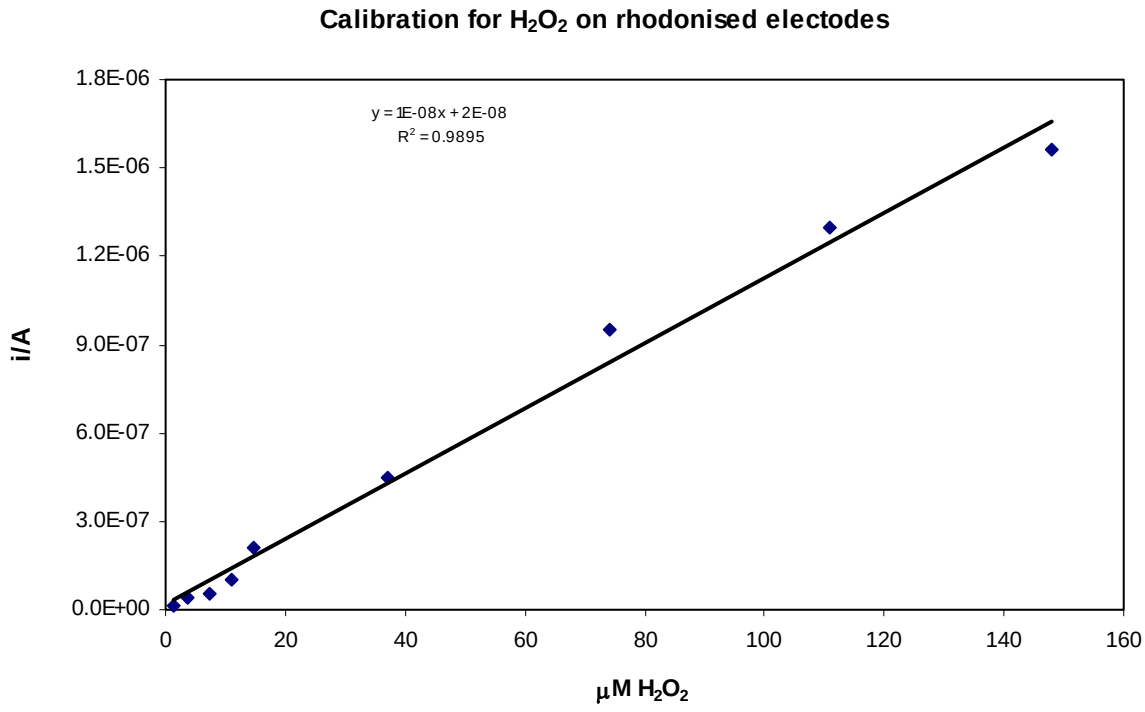


Figure 5.16: Detection of hydrogen peroxide on rhodonised electrodes at a potential of 300 mV.

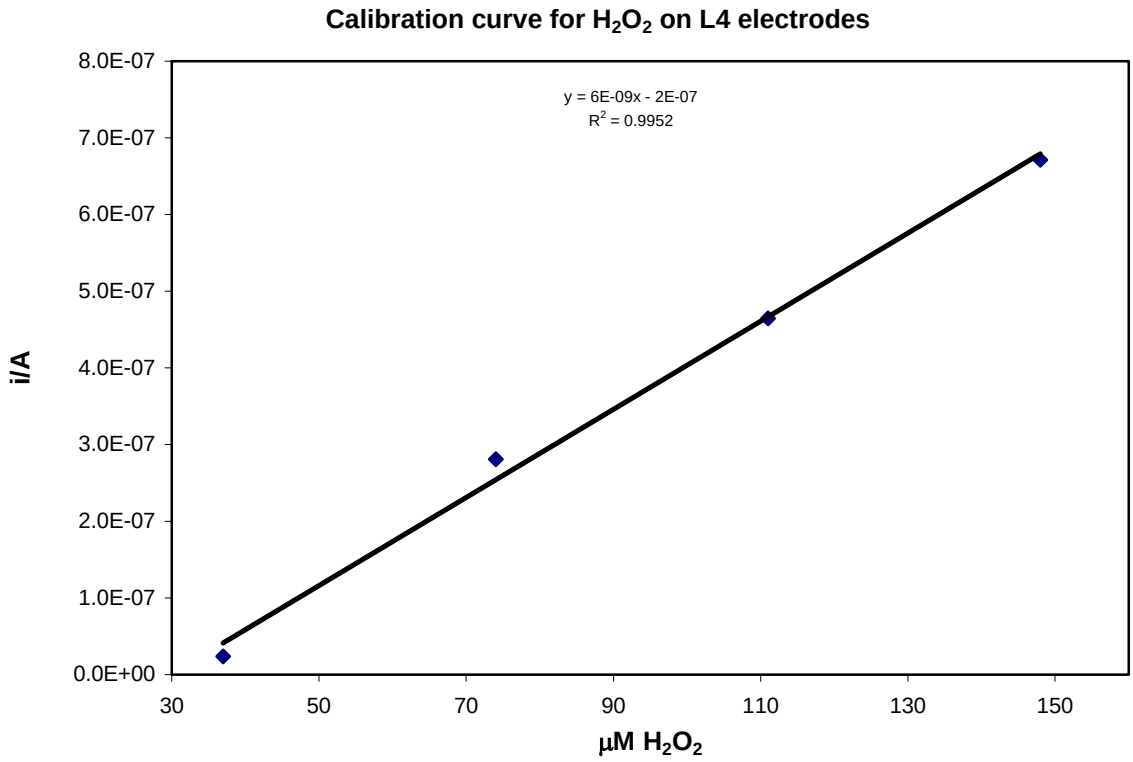


Figure 5.17: Detection of hydrogen peroxide on L4 electrodes at a potential of 600 mV.

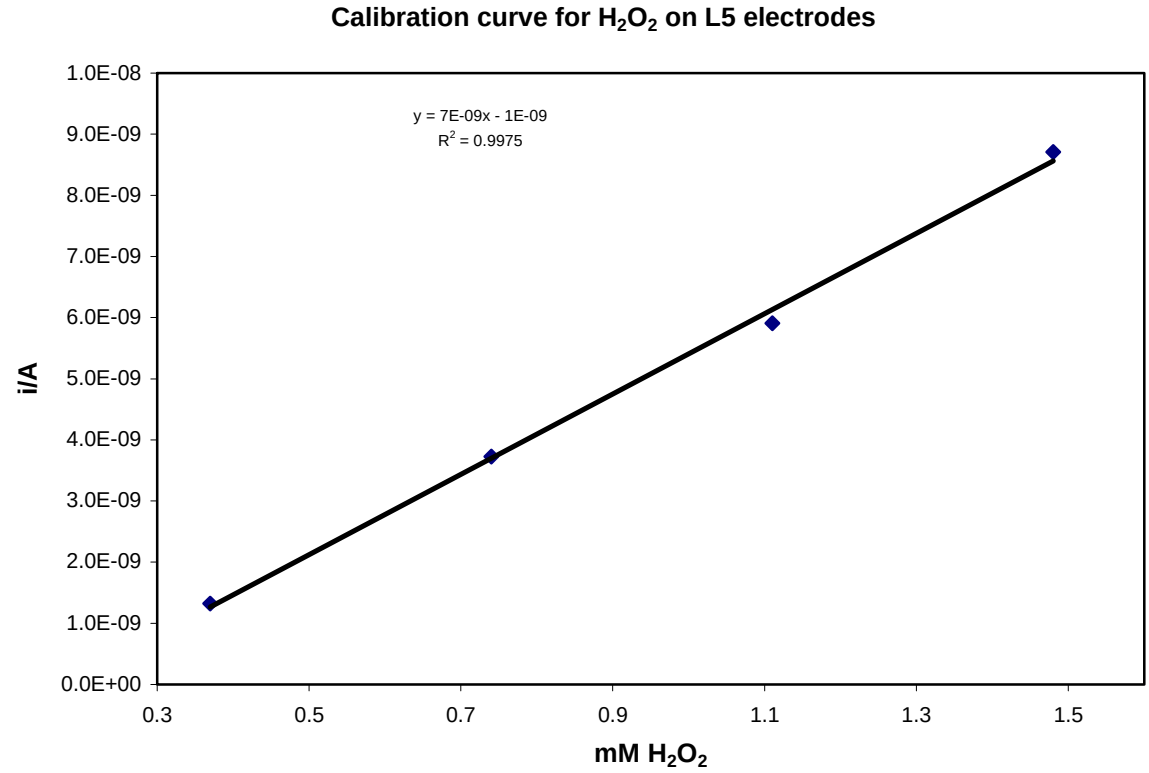


Figure 5.18: Detection of hydrogen peroxide on L5 electrodes at a potential of 600 mV.

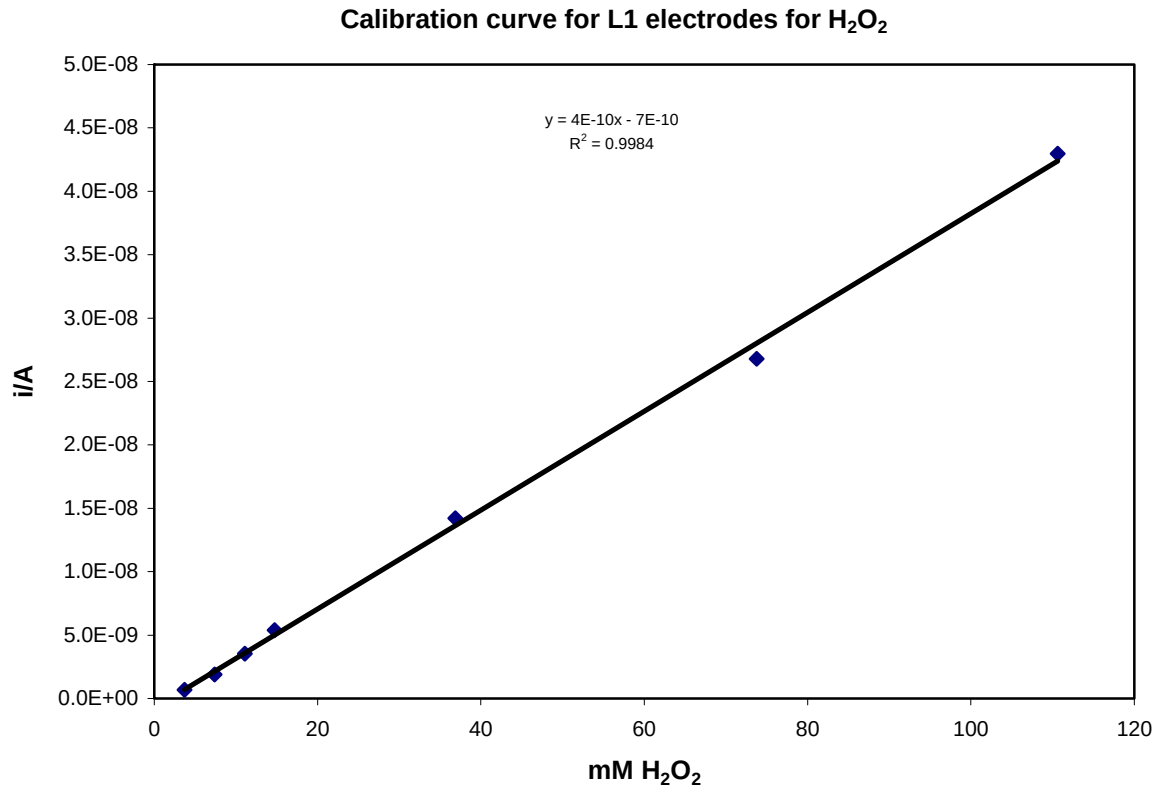


Figure 5.19: Detection of hydrogen peroxide on L1 electrodes at a potential of 650 mV.

Only L1 type of electrodes demonstrated a high enough sensor response with magnitude 3.7 μM (for 0.1 mM H_2O_2) (Figure 5.19). Even though carbon paste was printed on top of the platinum, and the potential was 650 mV, the reproducibility of these types of electrodes was acceptable (STD ~ 5%). Rhodonised and platinised carbon are able to lower the oxidation potential of hydrogen peroxide. Overall results of direct measurements of hydrogen peroxide are shown in Table 5.3. Unfortunately even though the potential of the rhodonised electrodes was low which is beneficial for minimising interferences, the signal response was quite noisy compared to other types of electrodes. We would expect however that a similar decrease in oxidation potential can be achieved by using an appropriate mediator (Cardosi and Birch, 1993).

Table 5.3: Overall calibration data from direct detection of H_2O_2.

Type of electrodes	Oxidation potential (mV)	Detection limit	Slope
Rhodonised	300	0.49 μM	$1E^{-08}$
L4 carbon	600	0.134 mM	$6E^{-09}$
Carbon	700	0.504 mM	$7E^{-08}$
L5 carbon	600	7.23 mM	$7E^{-09}$
L1 carbon/platinum	650	6.35 μM	$4E^{-10}$
Platinum	550	1.86 μM	$5E^{-09}$

5.3.4 Indirect amperometric detection of H_2O_2 with hydroquinone and HRP

Hydroquinone ($C_6H_6O_2$, solid, molecular weight 110.11 Da) was used to lower the detection potential for hydrogen peroxide and to increase the sensitivity of the sensor to enable its use in further practical applications. The electrochemistry of hydroquinone is well known and it has been a popular choice of mediator. Since it is a light sensitive compound, it needs to be protected from light and used quickly otherwise it degrades (giving a pale colour) and cannot be used in any measurements. It is a water-soluble compound, and it is important to ensure that it is present in sufficient concentration in solution especially if a flow cell is used.

Carbon electrodes were measured at the reduced potential of hydroquinone which was found to be at -300 mV (from CV data, see material in Section 5.3.1). The electrodes were dipped in the 40 mM phosphate buffer solution with the addition of 50 μl of 100 mM hydroquinone solution. By having enzyme and hydroquinone in solution the detection limit for the carbon electrode dropped from 1.48 mM to 0.405 μM (Figure 5.20). The amperometric response of the electrodes at the applied potential of -300 mV to the stepwise addition of H_2O_2 showed a large cathodic current corresponding to the reduction of hydroquinone that was generated enzymatically. This response became smaller with repeated additions of hydrogen peroxide. Electrodes were disposed of after

each measurement. This could not be performed with platinum electrodes as they are more expensive and would need to be regenerated for any practical sensor.

The polymer/carbon electrodes with enzyme and mediator in solution showed a limit of detection of 10.7 μM which was lower than that measured with plain carbon (Figure 5.21). This could be explained by the presence of the enzyme on the electrode surface due to covalent binding to thioacetale polymer.

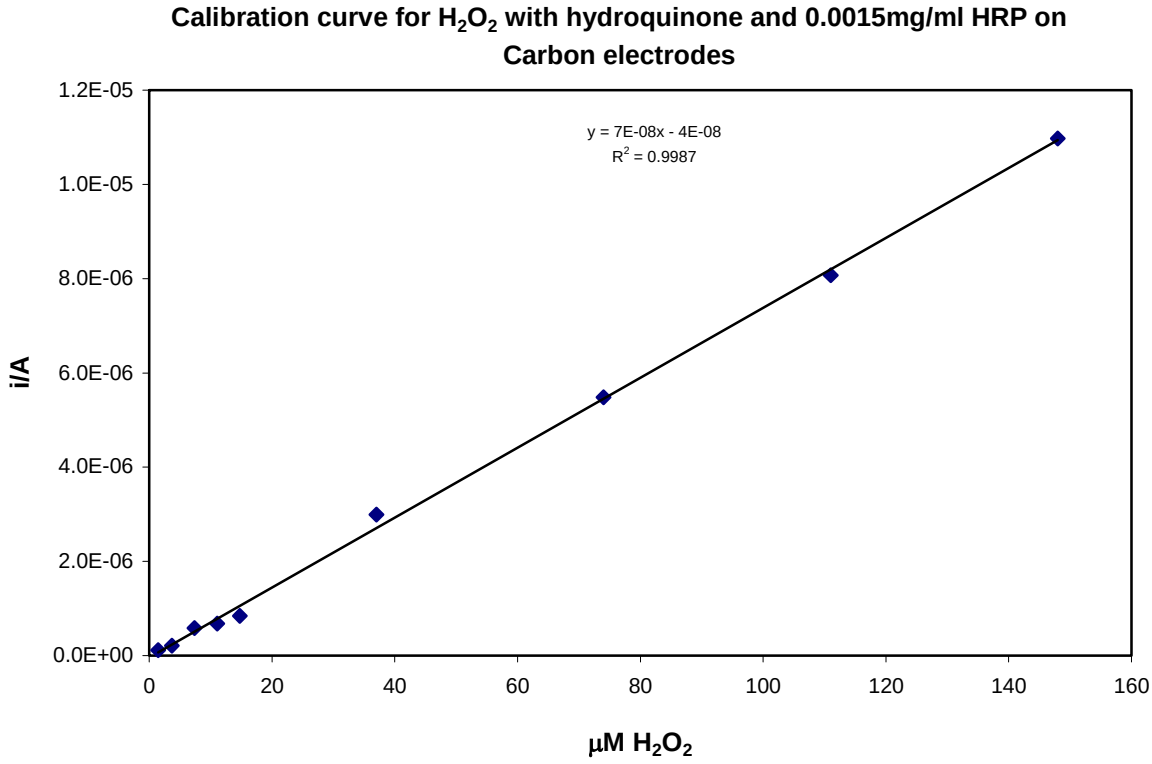


Figure 5.20: Detection of hydrogen peroxide on carbon electrodes with addition of 100 mM hydroquinone and 0.0015mg/ml HRP.

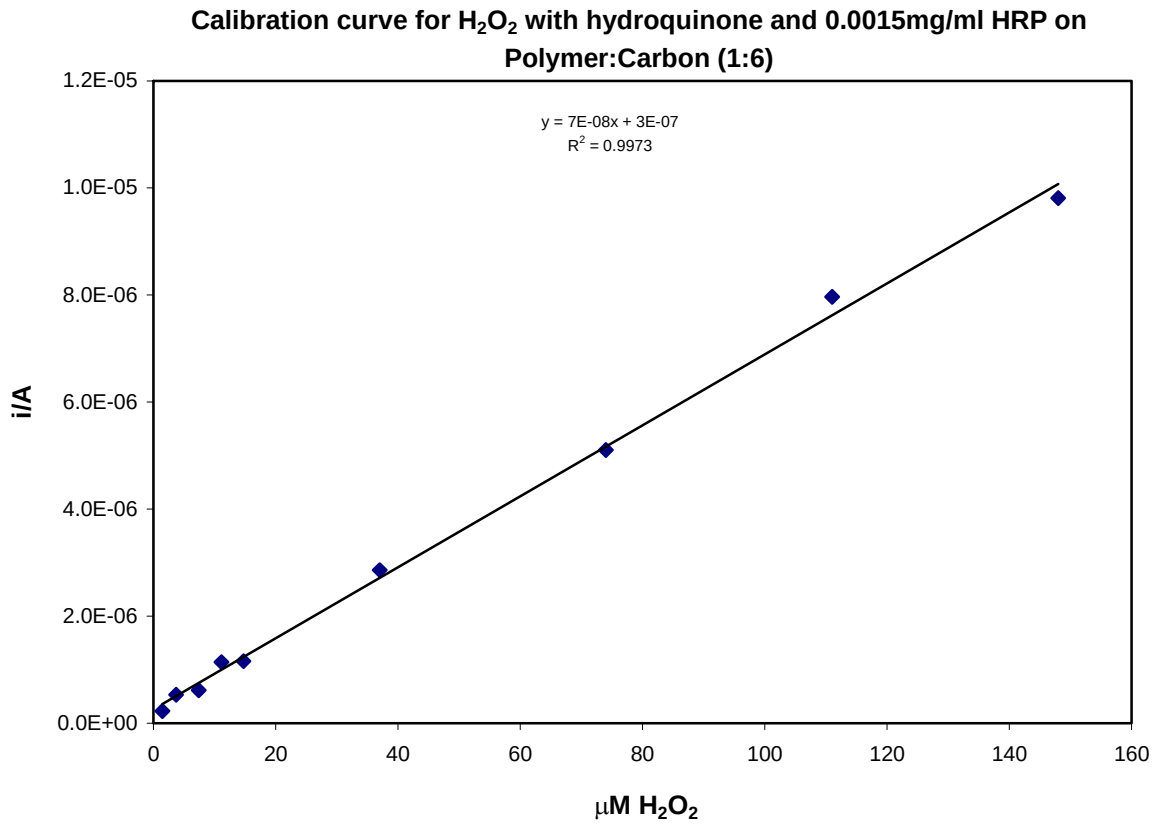


Figure 5.21: Detection of hydrogen peroxide on carbon/polymer electrodes with addition of 100 mM hydroquinone and 0.0015mg/ml HRP.

Calibration of rhodonised electrodes using 0.0015 mg/l HRP and hydroquinone gave a lower sensitivity (Figure 5.22) which shows that there is a need for amplification of sensor signal with enzyme for this type of electrodes. The electrodes (L1) demonstrated a worse performance with limit of detection around 8.98 μM (Figure 5.23). Their reproducibility was also quite poor. Carbon/polymer electrodes performed better than any other electrodes tested in this work. In all cases the sensor produced a kinetically controlled current linearly related to the hydrogen peroxide concentration within the range of 0.15 μM to 0.014 mM. The rapid loss of activity which is observed in the cyclic voltammetry of hydroquinone/HRP at the potential lower of -300 mV could be due to irreversible electroreduction of the HRP. At a concentration of enzyme higher than 0.0015 mg/l, the response signal was quite low due to depletion of substrate.

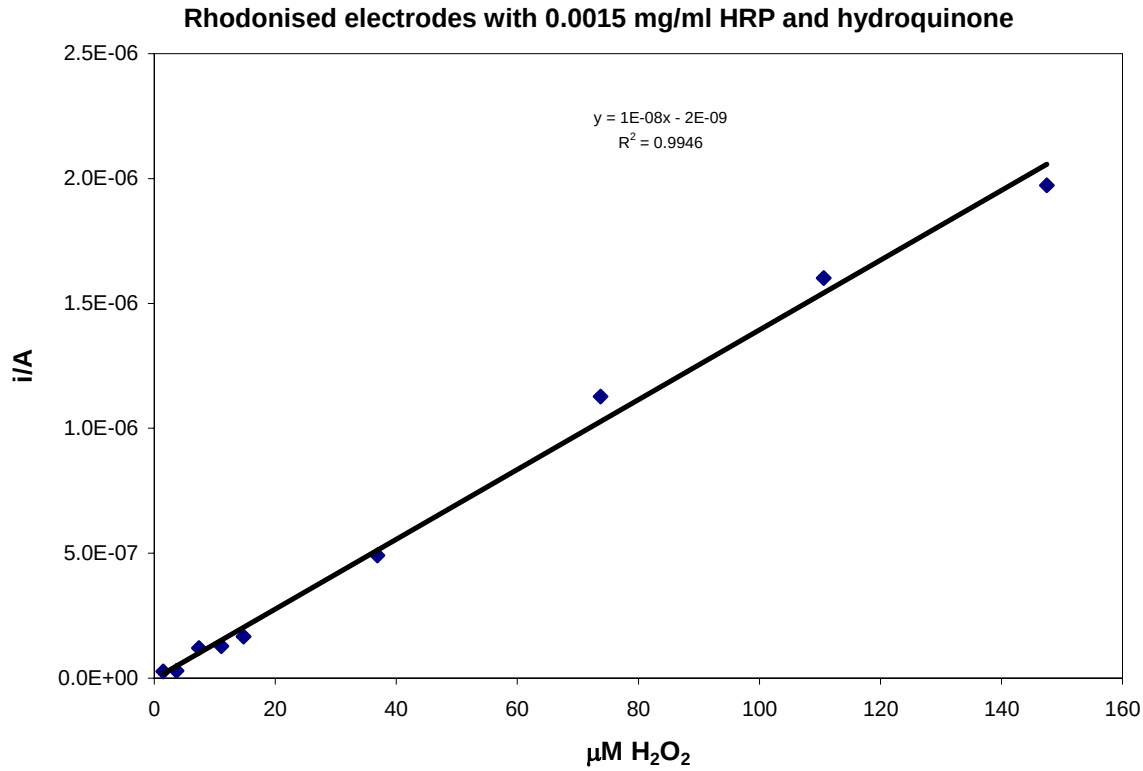


Figure 5.22: Detection of hydrogen peroxide on rhodonised electrodes with addition of 100 mM hydroquinone and 0.0015mg/ml HRP.

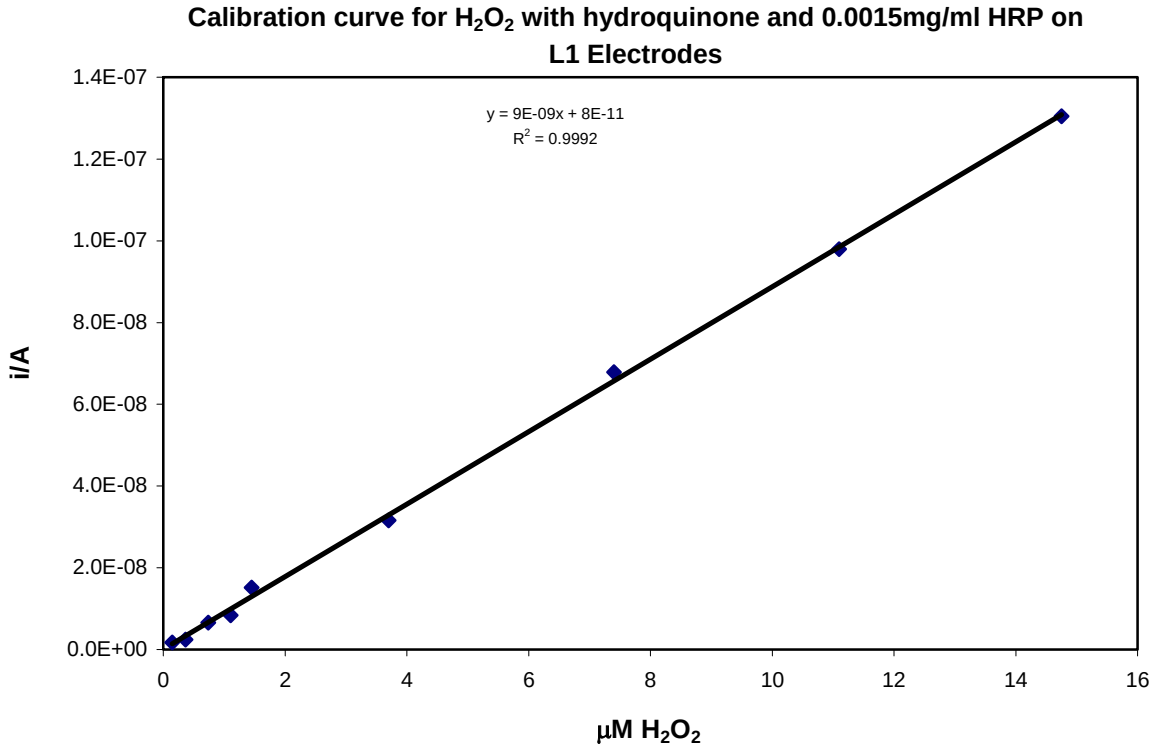


Figure 5.23: Detection of hydrogen peroxide on L1 type of electrodes with addition of 100mM hydroquinone and 0.0015mg/ml HRP.

Further experiments were performed by comparing physical absorption of the enzyme on carbon electrodes and covalent immobilisation on polymer/carbon ones. In both cases 50 μl of a 0.5 mg/ml solution of HRP was left to dry for an hour on the working surface of the electrodes. Electrodes were then rinsed with reverse-osmosis water and dried at room temperature prior to the measurements. The sensor response was measured in a 10 ml buffer solution containing 50 μl of hydroquinone. Samples with different concentrations of H_2O_2 were again injected using a micropipette and the chronoamperometric response from the reduction of hydroquinone was recorded. In this case polymer/carbon electrodes (Figure 5.24) indicated a higher sensitivity compared with plain carbon ones (Figure 5.25). This could be due to the better retention of the enzyme at the electrode surface. The polymer electrodes had a detection limit 0.766 μM compared to 2.05 μM achieved with carbon (unmodified) electrodes and adsorbed enzyme and 4 μM with carbon electrodes and enzyme in solution. Overall data for indirect detection of hydrogen peroxide are shown in Table 4.3.

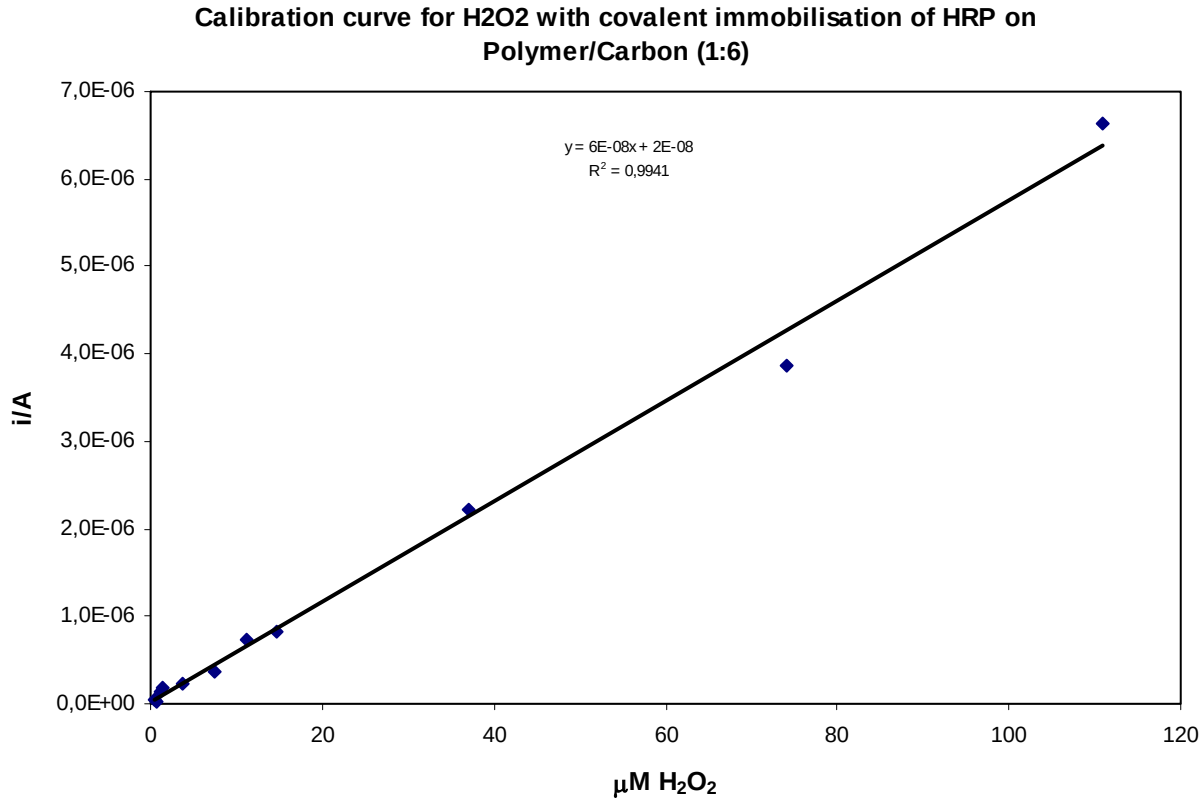


Figure 5.24: Calibration curve for H_2O_2 with covalent immobilisation of HRP on the surface of carbon/polymer electrodes.

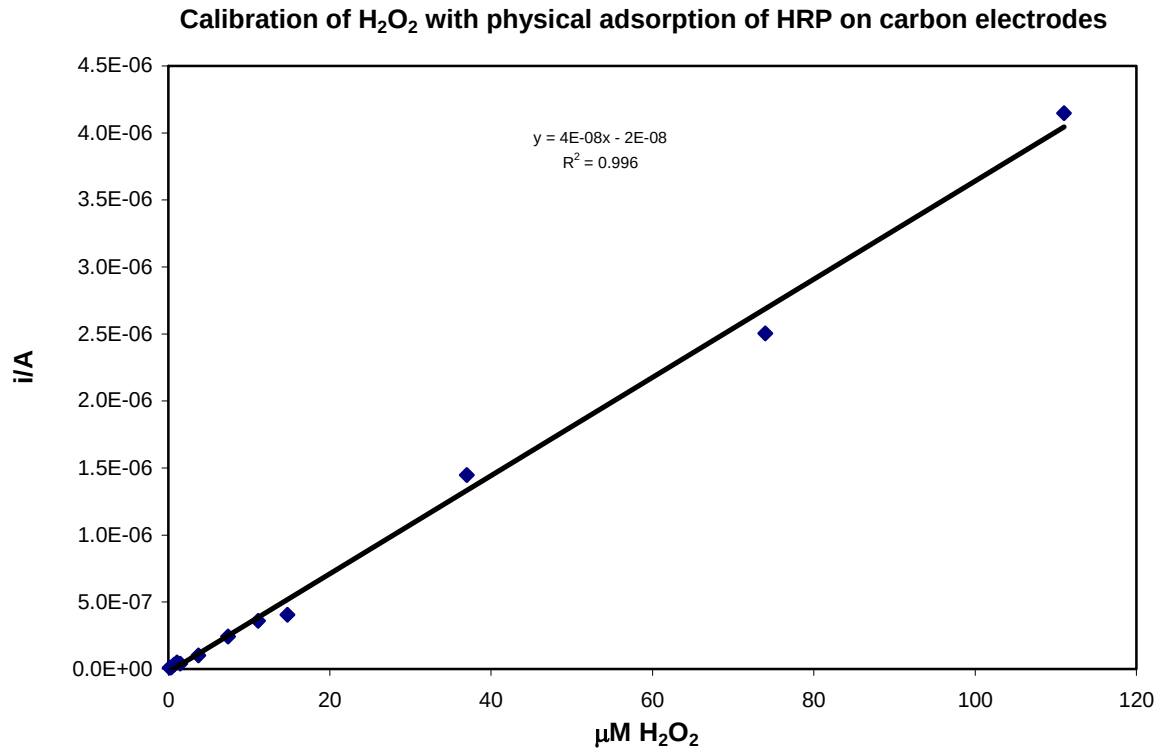


Figure 5.25: Calibration curve for H_2O_2 physical adsorption of HRP on the surface of carbon electrodes.

In cases where the enzyme was freely diffusing in the solution, carbon electrodes did not show comparable sensitivity to metallised ones (Tables 5.3, 5.4). Still the use of the reduction potential of hydroquinone (-300 mV), helps to avoid interference, which is pronounced at higher potentials. For further measurements the electrodes chosen were (i) carbon/polymer electrodes with immobilised enzyme and (ii) plain carbon electrodes. Testing from this point on was performed to identify the possible interference of chloroplast components into measurements of hydrogen peroxide using these two electrodes (see Chapter 6). With HRP immobilised on both electrode surfaces was further evaluated using flow injection analysis. This technique was used to optimise the system for the final step which is the detection of herbicides with chloroplasts/thylakoids from higher plants.

Table 5.4: Overall calibration data from indirect detection of H_2O_2.

Type of electrodes	Ox/Red potential (mV)	Detection limit	Slope
Platinum (L1) with hydroquinone and HRP in solution	-300	1.09 μM	$9E^{-09}$
Rhodonised with hydroquinone and HRP in solution	-300	0.205 mM	$1E^{-08}$
Rhodonised with HRP in solution (no hydroquinone)	300	6.8 μM	$5E^{-08}$
Rhodonised without HRP (no hydroquinone)	300	0.49 μM	$1E^{-08}$
Carbon/polymer with hydroquinone and HRP in solution	-300	10.7 μM	$7E^{-08}$
Carbon with hydroquinone and HRP in solution	-300	4.05 μM	$7E^{-08}$
Carbon/polymer with hydroquinone and HRP covalently immobilised	-300	0.112 μM	$6E^{-08}$
Carbon with HRP physically adsorbed	-300	2.05 μM	$4E^{-08}$

5.3.5 Flow-injection analysis

Automation of environmental analysis samples is an essential technique used in for preparation/dissolution of samples, determination of analyte content and even collation/reporting of results. It has been previously demonstrated that both manual and automated methods give similar results (Nausch, 1997). However, there are several advantages associated with automated techniques. These advantages are the capability for multi-analysis of species/elements, measurement of small sample volume tests, and the simplicity of incorporating of the technique with laboratory information systems that would be able to identify the samples automatically throughout the analytical procedure (Edwards *et al*., 2004). For example, automated systems for seawater analysis could produce a vast amount of data so that the automated handling is essential (Whitehouse and

Preston, 1997). The first continuous flow systems were introduced in 1957 by Skeggs and was used later in 1975 for environmental analysis (Růžička and Hansen, 1975; Ferreira *et al*., 1998).

Flow injection analysis in its simplest form is based on the rapid injection of a sample solution into a non-segmented carrier stream of reagent. It has similarities to high performance liquid chromatography (HPLC). Initially the sample distribution is controlled by convection, along with diffusion which becomes dominant over the distance. In practice, both convection and diffusion play an important part in the dispersion of the sample through FIA.

5.3.5.1 FIA for H_2O_2

For hydrogen peroxide detection through FIA the enzyme was physically attached to the electrode surface by depositing 50 µl of HRP solution (0.5 mg/10 ml) for 1 hour at room temperature. Phosphate buffer containing 1 mM hydroquinone was used as a solvent. Injected samples (25 µl) consisted of phosphate buffer, and hydroquinone spiked with different concentrations of hydrogen peroxide. Thus, further dilution of the sample occurred the minute it was injected in the buffer. Additionally, the length of the tubes was sufficient for H_2O_2 dispersion and accurate measurement when it reached the electrode surface. Low concentrations of H_2O_2 were used to prevent inactivation of the horseradish peroxidase. Injections were performed regularly allowing a buffer wash between each measurement. A typical response is shown in Figure 5.26 with each peak representing a hydrogen peroxide standard.

Six concentrations of H_2O_2 were detected amperometrically in the range from 1 to 120 µM. The calibration curve generated from the mean responses of three different sensors after the baseline responses had been deduced is shown in Figure 5.27. The limit of detection was found to be 4 µM for carbon electrodes and 1 µM for carbon/polymer ones, respectively. Reproducibility between the electrodes was good (RSD approximately 5%). From the Figure 5.27 it could be seen that the sensor response was linear at concentrations ranging from 14.5 µM to 0.1 mM. From the linearity at this range, the limit of detection was calculated. In the flow cell, the sensor responses at higher concentrations

of hydrogen peroxide were about 10% of that seen previously when the sensors were in a beaker of stirred buffer. In the flow cell, there is a continual renewal of the diffusion layer at the electrode surface compared to the beaker. Thus, equilibrium might never be reached in the flow cell compared to the stirred solution in the beaker where the sensor will not be as vigorously agitated and therefore closer to equilibrium. However the limit of detection from both cases is similar and the small deviation could be attributed to internal errors of the variation the sensor system.

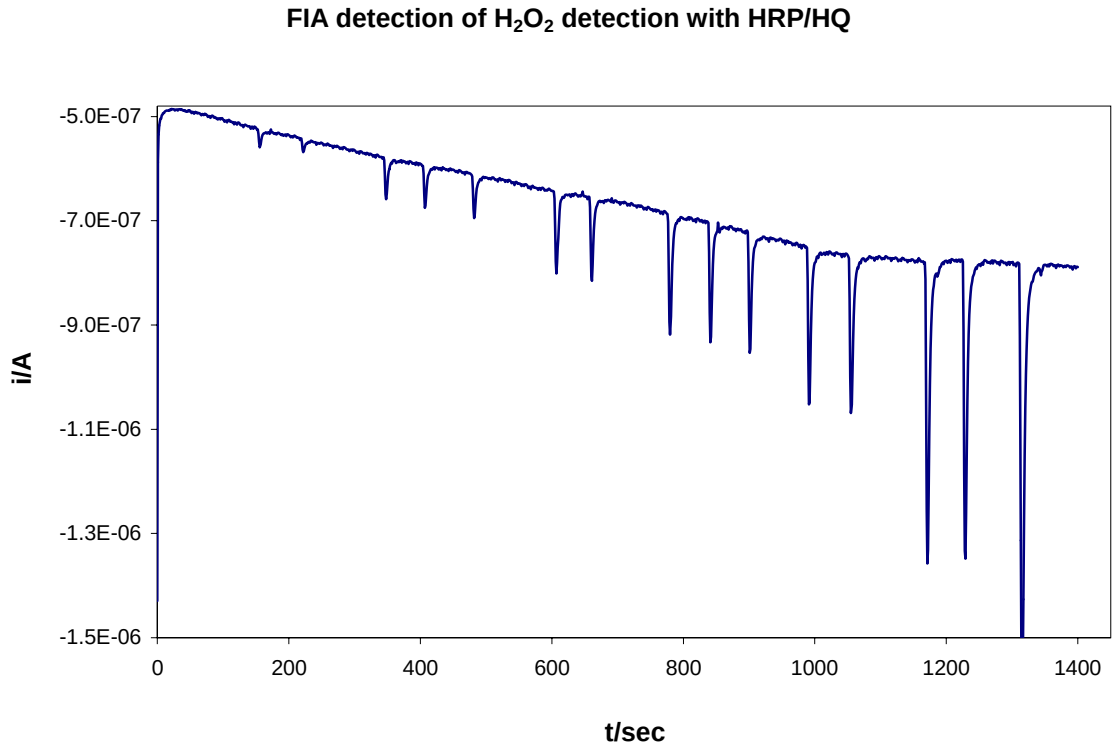


Figure 5.26: Sensor responses to a series of hydrogen peroxide standards.

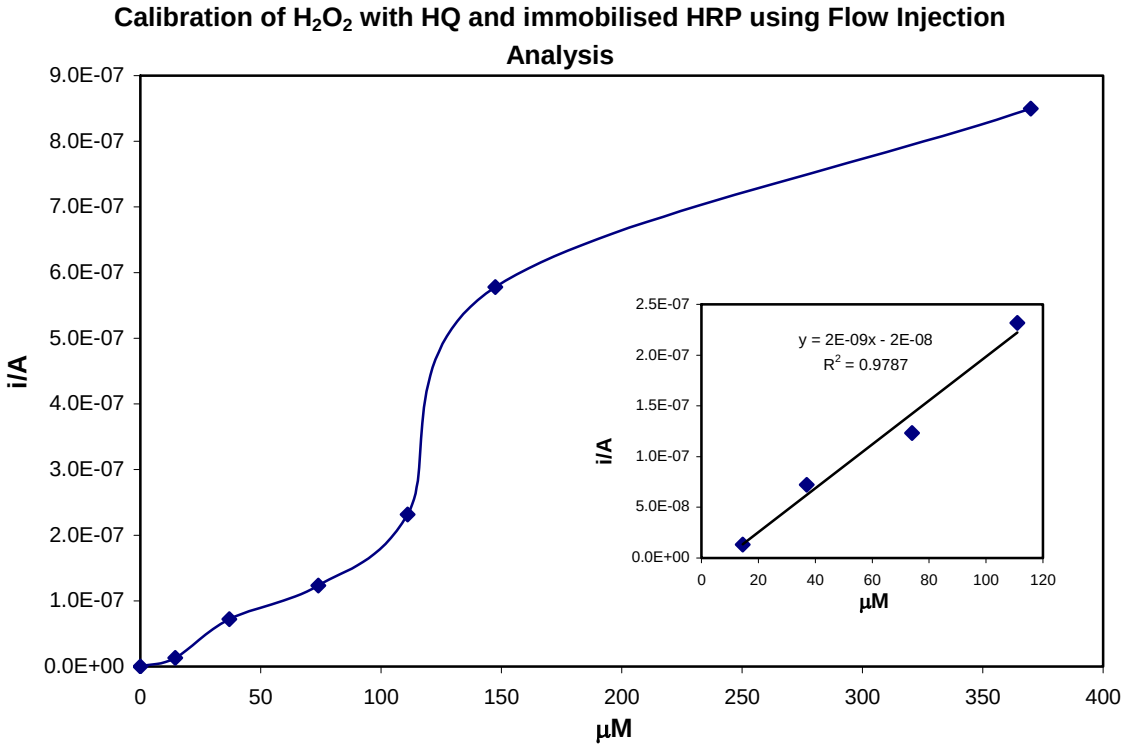


Figure 5.27: Calibration curve for hydrogen peroxide response with the sensor in the flow cell.

5.3.5.2 Stability testing

The sensor stability was tested for a period of 2 months. Experiments were performed at room temperature (25 ^{0}C). Each sensor was allowed to equilibrate to the room temperature and the temperature of the buffer until a stable baseline was observed. The lower concentrations of hydrogen peroxide were injected only, since at higher concentrations, the calibration solution would not fall in the linear range. There was small variation between the sensors tested on different days. Figure 5.28 indicates the carbon sensor response in the period of a day to 2 months. Similarly Figure 5.29 shows the response of carbon/polymer sensor.

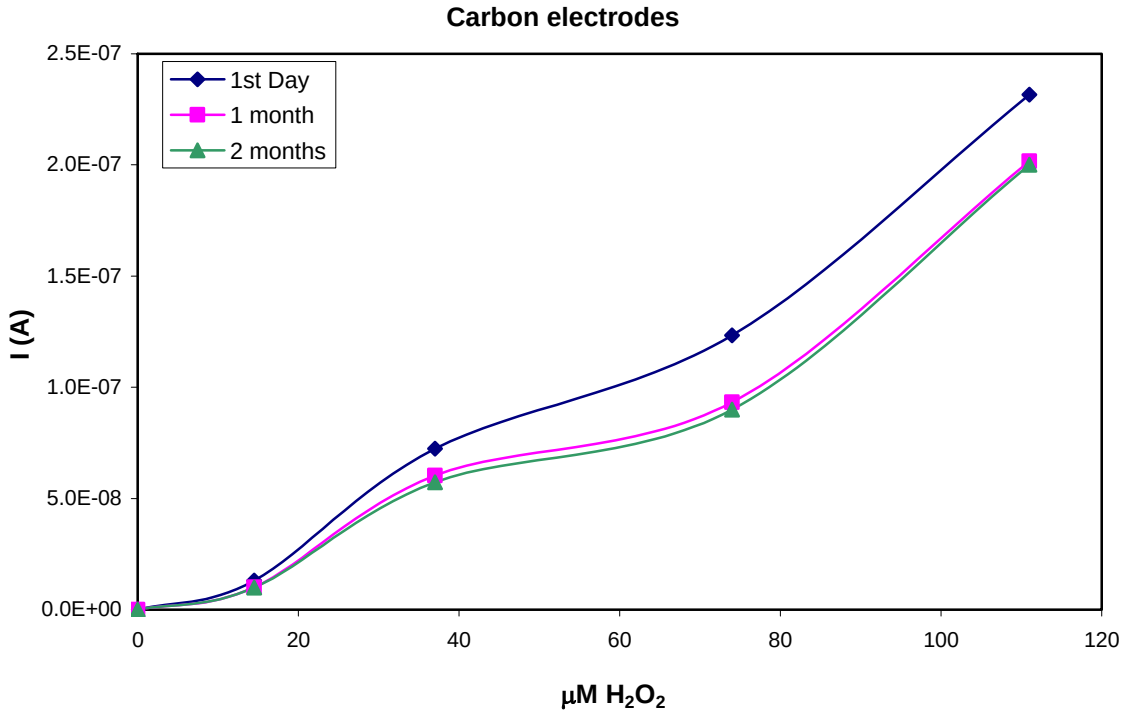


Figure 5.28: Comparison of carbon sensor response when fresh and after 1 and 2 months in the fridge at 4 ^{0}C.

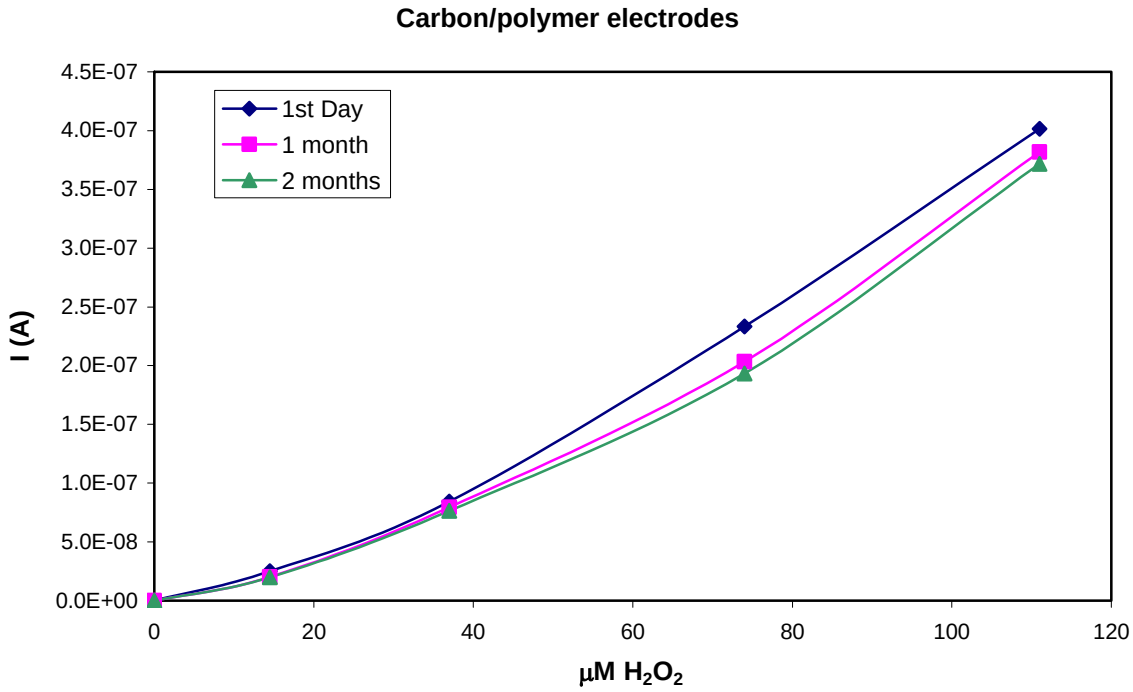


Figure 5.29: Comparison of carbon/polymer sensor response when fresh and after 1 and 2 months in the fridge at 4 ^{0}C.

Similarities of these data with those performed in a beaker of stirred buffer indicate a satisfactory limit of detection from both techniques. Furthermore, the peaks at flow cell analysis were clearer and broader, assuming that no inactivation of horseradish peroxidase was occurring. It has been previously reported that an extended limit of detection could be reached in flow cell analysis provided that the analyte dilution is small. The fact that a more sensitive detection was not reached through flow cell analysis could be due to larger dilution of the analyte. The slight decline of the sensors performance seen over the period of 1 to 2 months could be due to leaching of the enzyme or the mediator, or loss of enzyme activity in the fridge and/or electrode fouling. In a routine use of the technique these factors must be further investigated. The advantage of using the flow cell arrangement lies in the automation of the technique which could be exploited in future work.

5.4 CONCLUSIONS

In this type of work several electrodes have been tested which were either produced in the facilities of Cranfield University or purchased from commercial providers. As a result of all the measurements the feasible approach for development of electrochemical sensor for herbicides could be the one based on carbon electrodes as a matter of simplicity, used either in stationary way or in a flow cell system. Carbon/polymer could be also used for future research in the development of enzyme sensors but they were not necessary for the production of cell based sensors used in following work. Metal electrodes and the ones containing metallised paste (rhodonised) were excluded from further work for the same reason.

Results from flow injection analysis demonstrated sensitive detection of hydrogen peroxide. This arrangement has been shown to give better reproducibility and therefore was used as a more appropriate analytical procedure for further measurements. The degree of automation offered by this technique will also allow extended measurements, which in the case of herbicide detection is essential as the time of analysis required is longer than the one of hydrogen peroxide.

CHAPTER 6. DESIGN OF CHLOROPLAST SENSOR

6.1 INTRODUCTION

In previous chapters, the components suitable for the production of the sensor were selected according to literature and the results of our experiments, aimed at detection of H_2O_2 with screen-printed electrodes. The final selection of electrodes was based on relative performance and consideration of the cost of electrode manufacturing. FIA was chosen as the final analytical procedure, which allows the degree of automation necessary for future work. This chapter describes our initial attempts to design chloroplasts based sensors where the hydrogen peroxide sensitive electrode (with HRP) is combined with chloroplasts in an illuminated flow cell. The idea is to illuminate chloroplasts which will produce hydrogen peroxide, which in turn will be detected by H_2O_2 sensor, using hydroquinone and HRP. In addition to this reaction, an alternative approach has been studied based on the Hill reaction. This reaction is inhibited by herbicides and as such could be used to detect them in environmental samples. Preliminary work on the Hill reaction using screen-printed electrodes is also shown.

The properties of the photochemical apparatus need to be carefully considered according to the reactions taking place and the monitoring process. Photoelectrochemical cells were used mainly to study biochemical processes involved in photosynthesis, such as determination of the sequence of the light and dark stages of photosynthesis and elucidation of molecular mechanisms of key reactions of electron and photon transport in chloroplasts. Photoelectrochemical cells have been used in the past as an alternative approach for monitoring the pseudocyclic electron flow in chloroplasts/thylakoids (Allen and Crane, 1976; Lemieux and Carpentier, 1988; Goetze and Carpentier, 1990). In this work chloroplasts and/or thylakoids were used either in suspension or immobilised on the electrode surface (Koblitzek *et al*., 1998; Touloupakis *et al*., 2004). The measurements were performed at wavelengths where the spectral difference between the oxidised and reduced forms of the photosynthetic pigments and/or of substances which take part in the electron transfer chain (P700, cytochromes, plastocyanin, plastoquinone, ferredoxin, etc.) were maximal. Since the difference in these wavelengths could be extremely small and transient, their monitoring requires relatively complicated apparatus and most of the times

specially pre-treated plant material such as isolated cell layers, solvent treatment, buffer requirements, etc (Šesták *et al*., 1971).

In the present work we developed a photoelectrochemical sensor consisting of two main elements: a photochemical flow cell and electrochemical sensor based on a screen printed electrode. We discuss here also thylakoids and the mediator used for generating sensor response.

6.2 COMPONENTS SELECTION

6.2.1 Flow cell device

The first part of the work was related to the construction of a flow cell system. For the purpose of the project, the flow cell has to accommodate LED light sources for the illumination of chloroplasts and the electrochemical sensor. A flow cell device was provided by Das Srl, Italy. The flow cell device is a box with a size of 20x15x13 cm and weight of 1 kg. The device consists of three black Perspex flow cells where sealed printed electrodes can be inserted. Each cell has 2 flow ways for fluid movements: 1 flow input and 1 flow output. The volume of the flow-cell was adjusted by a silicon spacer (0.5 mm) (Krejici Eng. Czech Rep). This device incorporated three LED (Figure 6.1) having wavelengths 800 nm, 650 nm, and 430 nm (Figure 6.2). Light was directed towards the working surface of the screen-printed electrodes. The parameters of the LED sources are presented in Table 6.1. These are related to the working electrode surface area of the screen-printed electrodes.

Figure 6.1: High brightness LED lamp (visible wavelength).

Figure 6.2: Illuminator with 3 different LED sources.

Table 6.1: LED properties for flow cell device.

Properties	Value
Emitting colour	Red, blue or near infra red
Lens colour	transparent
Lens size (mm)	3
Dominant emitting wavelength (nm)	650 (red), 430 (blue), 800 (non-visible)
Viewing angle (degrees)	10

Changes of the current intensity on the carbon electrode were registered by the same potentiostat (μAutolab Electrochemical Analyser) as used in Chapter 3. No special electrochemical treatment of the electrode surface was used. The electrodes were washed with RO water and purged with nitrogen (N_2 gas) to remove any dust accumulation.

For the measurements of the chloroplast signal using the H_2O_2 sensor, the microflow was connected to a Hewlett Packard 1050 pump which has a maximum pressure of 8 bar (5ml/min). During measurements the flow was set to 0.5 ml/min at the pressure of 1 bar. With lower flow, mixing should be more homogeneous by the time it reaches the electrode surface. Conditions remained the same throughout the analysis of chloroplast electrochemical signal. The electrodes used for this analysis were the ones

prepared at Cranfield University. This type of electrode has demonstrated a detection limit of 0.4 μM H_2O_2 when used through flow injection analysis.

For the measurements of the Hill reaction, the flow cell was connected to a peristaltic pump and the flow rate was set to 1 ml/min. The peristaltic pump was chosen due to its simplicity and low cost. The Hewlett Packard 1050 pump tended to block with the chloroplast suspension running through the tubes. The peristaltic pump allowed a wide choice of tubing which could allow viscous solutions to run through efficiently. Again the carbon screen-printed electrodes were those used earlier.

6.2.2 Biological material

Two type of biological sensing material have been used in this study - the enzyme (HRP) and thylakoids extracted from spinach. The protocol of the HRP and hydroquinone reaction is as described in Chapter 4. Enzyme was physically adsorbed onto the electrode surface by depositing 50 μl of HRP solution (0.5 mg/10 ml) for 1 hour at room temperature. The phosphate buffer solution running through the system contained 1 mM hydroquinone. The chloroplast extraction was performed the using modified protocol described previously (Piletskaya *et al*., 1999): chloroplasts were isolated from fresh spinach leaves (*Spinacea oleracea L.)*. Leaves (100 g) were washed with water, dried on filter paper and homogenised in 300 ml of extraction buffer containing 0.35 M sucrose, 50 mM Tris-HCl buffer, pH 8.0 and 10 mM NaCl. The homogenate was filtered through 4 layers of cheese-cloth and through sieves with a pore diameter 100 μm. Centrifugation was performed for 10 min at 2 000 x g (Model J2-21 centrifuge Beckman, Germany). The pellet was re-suspended in extraction buffer containing 1% of BSA. The 200-μl aliquots were placed in Eppendorf tubes and kept at –80 ^{0}C. Chlorophyll concentration was measured and adjusted to 3 μg/ml.

The chloroplasts were used in suspension and the solution was continually stirred. This would allow a more homogeneous sample to run through the system. Several concentrations of chloroplasts suspension were tested in the system and the optimal one in terms of electrochemistry was found to be 400 μl in a 50 ml phosphate buffer.

6.2.3 Mediator selection

The initial choice of mediator has been made according to the results in Chapter 4, which showed a sensitive detection of H_2O_2 using hydroquinone, measured at a reduction potential of -300 mV. For this work, 50 μl of 100 mM of hydroquinone were injected in the electrochemical cell containing thylakoids and HRP physically adsorbed on the electrode surface. In a separate experiment 2,6-dichlorophenol indophenol was also employed as a mediator to ensure integration of the Hill reaction with the electrochemical sensor. This mediator is able to penetrate quickly into the cell; it is electrochemically active and its reduced form is quite resistant to oxygen (Kuznetsov *et al*., 2004). Both mediators were used in a phosphate buffer of pH 7.4 containing 0.1 M KCl.

Cyclic voltammetry was performed on the electrodes having chloroplasts in suspension and DCPIP as the mediator. This was performed in a phosphate buffer pH 7.4 containing 0.1 M KCl. The solution was continually stirred and the concentration of DCPIP was set at 7.5 μM. The measurement was performed in the dark with and without chloroplasts in suspension. The potential chosen for the amperometric measurements of the Hill reaction was the one determined in the CV experiments with illuminated chloroplast suspension in the buffer.

6.3 RESULTS/DISCUSSION

6.3.1 Hill reaction

In the 1930s, a plant physiologist named Robin Hill demonstrated that isolated chloroplasts could evolve oxygen in the absence of carbon dioxide. During the Hill reaction, as it has been termed, oxygen is evolved by chloroplast fragments in the presence of an artificial electron acceptor. In the intact leaf cell, ferredoxin, NADP and, ultimately, 3-phosphoglyceric acid accept the electrons produced by photolysis. These factors were lost in Hill's isolation of chloroplasts, but he found that 2,6-dichorophenol indophenol (DCPIP) could be used as a substitute electron acceptor. Thus the reaction rate measured by the loss of blue colour is a measure of the activity of PSII under the given conditions of illumination (presence of substrates and inhibitors, etc.). DCPIP receives the electrons from herbicide-binding protein (D1 protein), more exactly, from its association

with D1 protein plastoquinone Q_B (Figure 6.3). Since the electron flow through PS II is affected by presence of herbicides, the reduction of DCPIP is proportional to herbicide concentrations and can be used for herbicide detection.

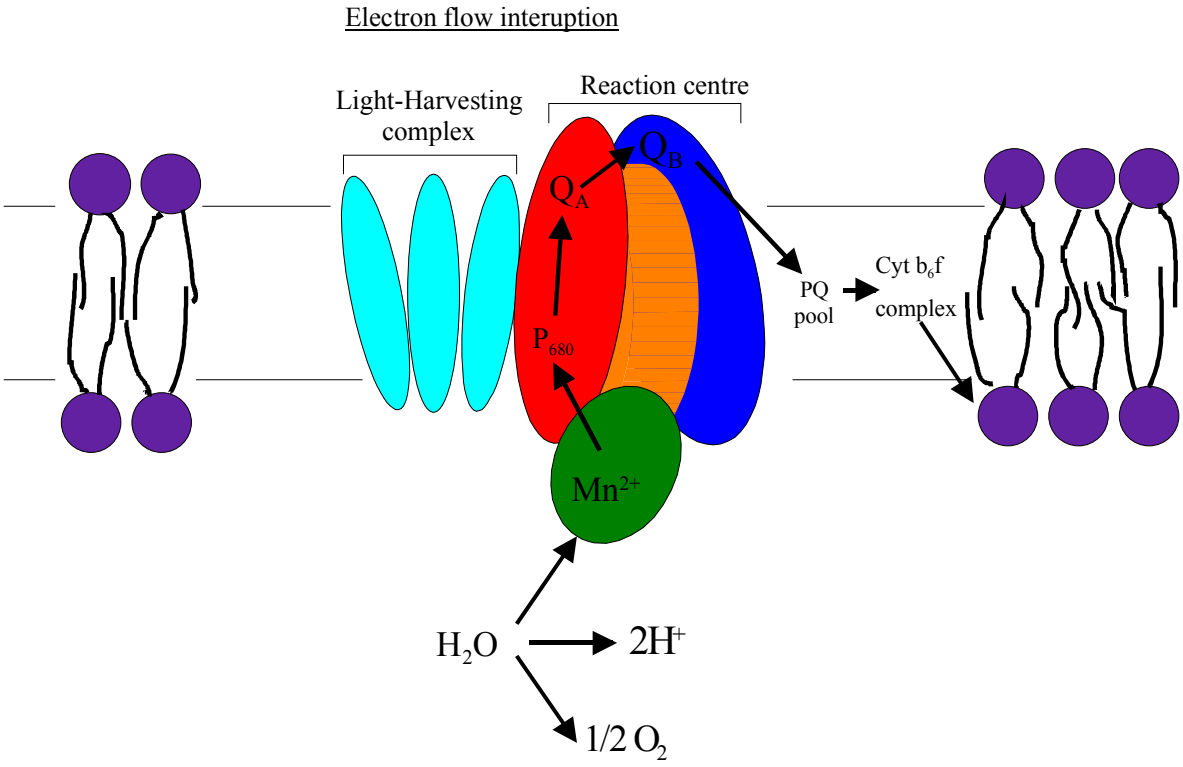


Figure 6.3: Scheme of photosystem II electron-transport chain.

The wavelengths of 650 nm and 430 nm, were selected for the chloroplasts measurements in this project. These wavelengths are related to chlorophyll a and chlorophyll b adsorption maxima (Figure 6.4). Chlorophyll acts as photoreceptor in the reaction of photosynthesis by trapping light. Its molecular structure is that of a porphyrin ring and resembles the structure of heme group found in hemoglobin, with only the exception of having a magnesium atom in the centre of the structure compared to the iron atom in the centre of heme. The two types of chlorophyll shown in the spectrum in Figure 6.2 differ only in the composition of their sidechain; chlorophyll a has a $–CH_3$ group and chlorophyll b has –CHO group (Figure 6.5). Both of these two chlorophylls are very effective photoreceptors because they contain a network of alternating single and double bonds, and the delocalised orbitals which stabilise the structure. The different sidechain structure of the two chlorophylls is what makes the difference in the absorption spectrum (Figure 6.6).

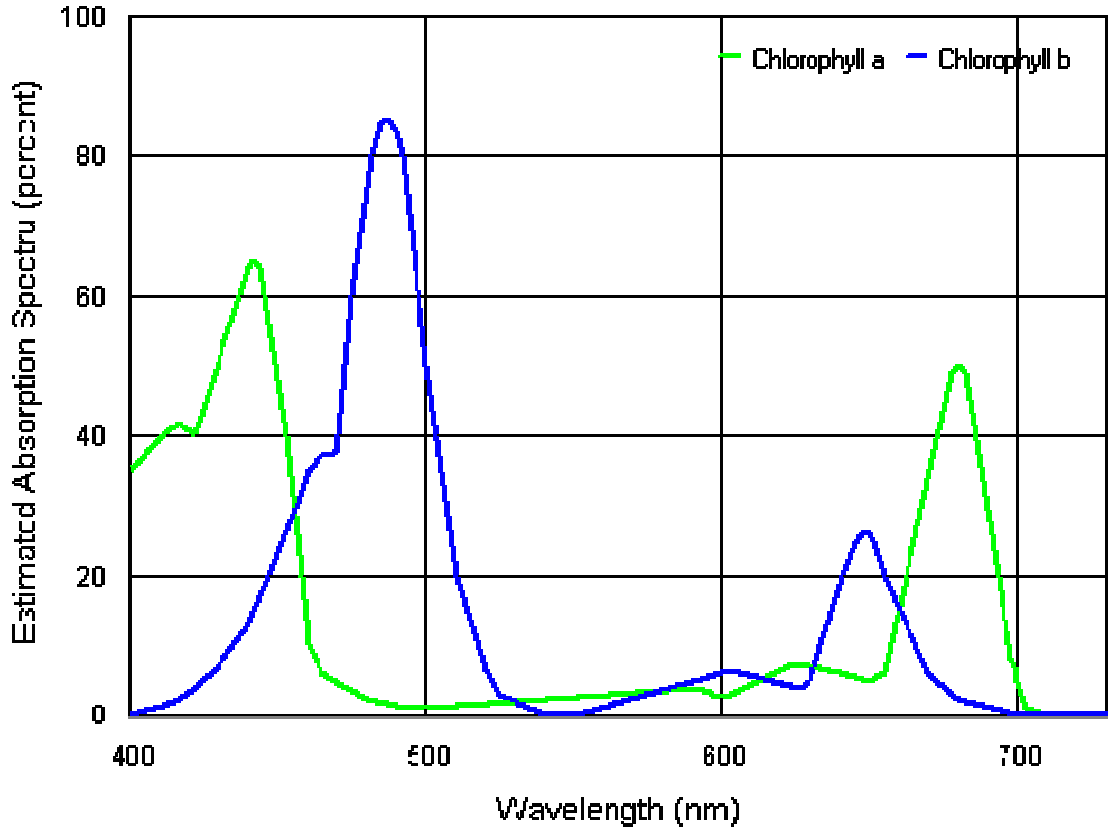


Figure 6.4: Absorption spectra for chlorophyll α and β (Gregory, 1971).

For example, light that is not entirely absorbed by chlorophyll a at the 430-460 nm wavelengths is absorbed by chlorophyll b, which has slight shift in maximum. Thus, the two chlorophylls complement each other in the absorption spectrum. Similarly, at the wavelength of 650 nm, chlorophyll a absorbs more light than chlorophyll b (Stryer, 1975). It is due to this knowledge that the wavelengths chosen for preliminary measurements in chronoamperometry were 430 nm and 650 nm.

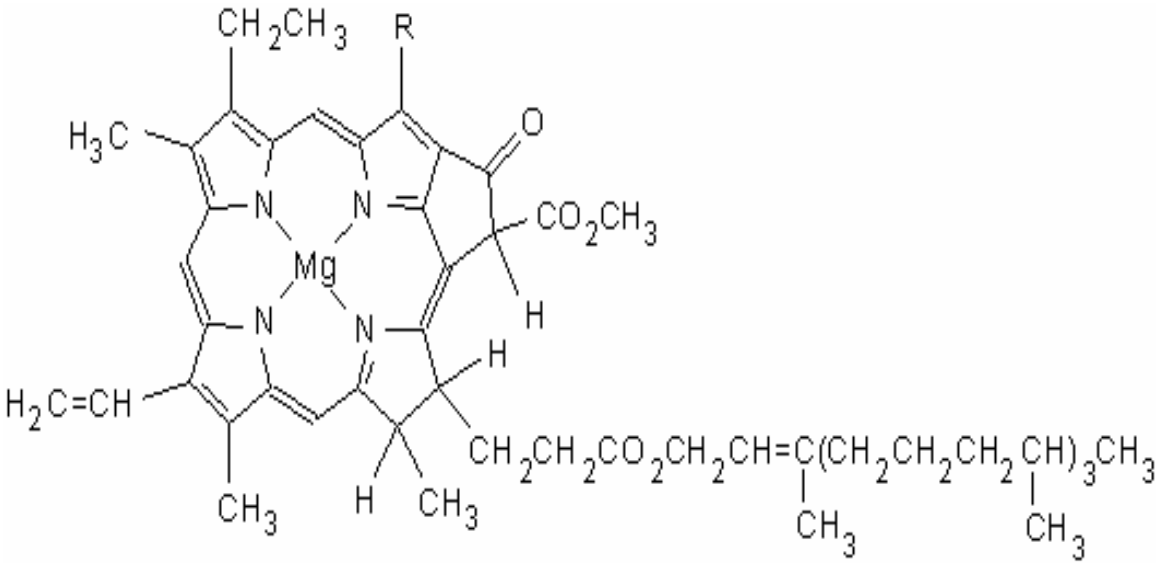


Figure 6.5: Chlorophyll molecular structure, where R is CH_3 for chlorophyll a and CHO for chlorophyll b.

6.3.2 Chloroplast signal using H_2O_2 sensor.

One of the important factors in the regulation of photosynthesis is the interaction of O_2 and components of the electron transport chain of chloroplasts, as this reaction is accompanied by the generation of oxygen active species such as $\cdot OH$, H_2O_2, $^1O_2$, $O_2^{\cdot -}$, which could damage the cells. In the chloroplast the photoinduced production of oxygen occurs in the protein photosystem I (PSI). At the acceptor side of PSI, oxygen interactions could lead to the production of superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide. In the past, interaction of photosystem II (PSII) and O_2 that also leads to the production of superoxide anion ($O_2^{\cdot -}$) and H_2O_2 has been studied and found taking place in the site near the PSII reaction centre (Zastrizhnaya *et al*., 1996). In our system this hydrogen peroxide was detected by an HRP-based biosensor. For matters of simplicity, no photosystem extraction occurred, and amperometric tests were performed using whole chloroplasts or thylakoids. The light absorbed by photosynthetic pigments is expressed as chemical energy and it is transferred at the reaction centre of photosystem I and II, where a charge separation takes place. The PSI complex in plants catalyses the oxidation of plastocyanin and the reduction of ferredoxin. Its reaction centre comprises of more than ten polypeptides and has similarities with the PSII reaction centre (discussed extensively in Chapter 7). The primary electron donor and acceptor in the PSI centre are bound to the

polypeptides *psaA* and *psaB* that form a heterodimer. In PSI the primary donor, P700 is a chlorophyll demer and the primary acceptor Ao, is a chlorophyll monomer. On the contrary, PSII has a single electron acceptor (A1) which is a quinone known as phylloquinone.

For assessing the H_2O_2 peroxide production, the resulting signal from the thylakoid illumination (Figure 6.6) was calculated and the current value I (Amp) was placed in the calibration curve for hydrogen peroxide (Chapter 5, Figure 5.27). The chloroplasts were freely suspended in a buffer solution, which also contained hydroquinone, and physically absorbed enzyme (HRP). Amperometric data with thyalokoids illuminated for approximately 5 minutes showed just a small signal (ΔI/A=8.62426E-08). This current falls in the range of 0.121 mM H_2O_2 concentration. Some inhibition from catalase could have occurred which could explain the reduced signal but only if the hydrogen peroxide concentration is high enough to be effectively degraded by this enzyme. At low concentrations of hydrogen peroxide (like the one here), catalase could not be the removal mechanism due to its low affinity for this substrate. A proper candidate for H_2O_2 removal would be ascorbate peroxidase, which catalyses the reaction:

$$H^{2+} + ascorbate + H_2O_2 \rightarrow dehydroascorbate + 2H_2O \qquad (6.1)$$

and that has been detected in spinach chloroplasts in concentrations up to 50mM (Halliwell, 1984).

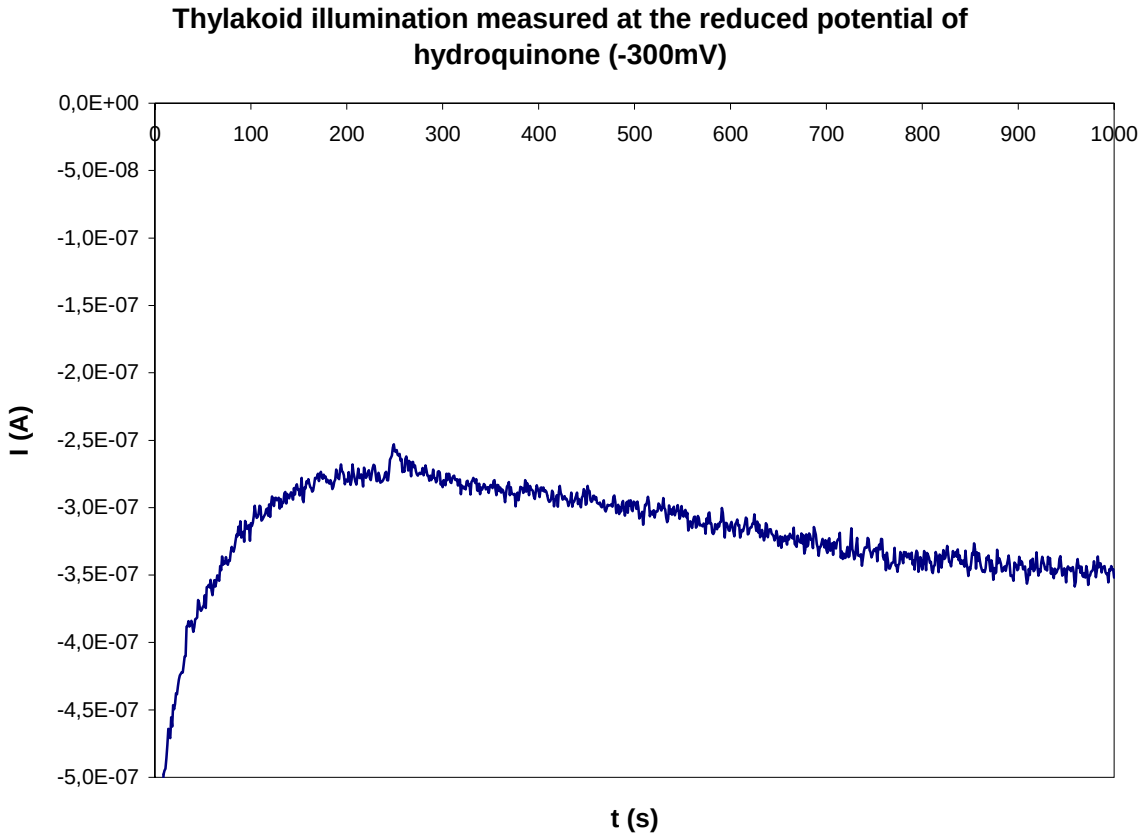


Figure 6.6: Thylakoid illumination at the reduced potential of hydroquinone (-300 mV), having HRP physically adsorbed.

The concentration of H_2O_2 was sufficient to be calculated but too low for reliable flow injection analysis. In the past it has been discovered that methyl viologen induces the photoreduction of dioxygen by accepting electrons from the iron-sulfur cluster of PSI and therefore accelerates the production of H_2O_2 (Fujii *et al*., 1990). In theory the signal for H_2O_2 can be increased by three times by adding methyl viologen to the solution. This still would not be enough since the sensor has a limit of detection of 4 µM (see Chapter 4). Since the resulting signal generated by H_2O_2 was insufficient, the system had to be modified. Thus, for the continuation of the measurements, the Hill reaction using DCPIP was chosen as it is a proven and successful existing technique. In this study the mediator – DCPIP has been detected electrochemically.

6.3.3 Electrochemical variant of Hill reaction

In this chapter we discuss the selection of excitation wavelength. The electromagnetic spectrum that is visible by a person (in the region of 400 nm to 700 nm) is the one also exploited by plant and algae for the process of photosynthesis. This radiation band used for photosynthesis is not in the invisible near infrared region which most photosynthetic bacteria or other physiological processes use. As it was mentioned before the region where illumination of chloroplasts took place for this preliminary work was 430 nm and 650 nm produced by blue and red colour LEDs (Light Emitting Diodes). The beam of light is a flow of energy which is not continuous but arrives in a series of packets known as quanta. For plant photosynthesis this flux of quanta is commonly measured in μmoles m^{-2} s^{-1} (Harbison and Rosenqvist, 2003). The energy of this quantum (photon) is calculated by the following equation:

$$E = hv \tag{6.2}$$

where E is the energy of the quantum, h is the Planck's constant (6.63 x 10^{-34} Js) and v is the wavelength of the light. The calculated energy applied in this work is 3.06 x 10^{-19} J for the 650 nm wavelength (red light) and 4.6 x 10^{-19} J for the 430 nm (blue light).

The electrochemical measurements in this work are associated with the electron flow generated in the chloroplasts of higher plants which originate from the absorption of light. This was accomplished using DCPIP as the mediator in an attempt to develop an electrochemical version of Hill reaction. The measurements were performed in buffer solution (40 mM phosphate buffer pH 7.4) containing chloroplasts with chlorophyll values of 30 ng/ml and DCPIP (7.5 μM). Measurements were performed in a dark environment and chloroplast concentrations were defrosted in cold water for 5 minutes. The chosen electrochemical potential results from the cyclic voltammetry data of DCPIP (Figure 6.7) at scan rate of 50 mV. The response time of DCPIP started at 100 seconds and the electro-reduction started at + 0.2 Volts having chloroplasts under illumination conditions.

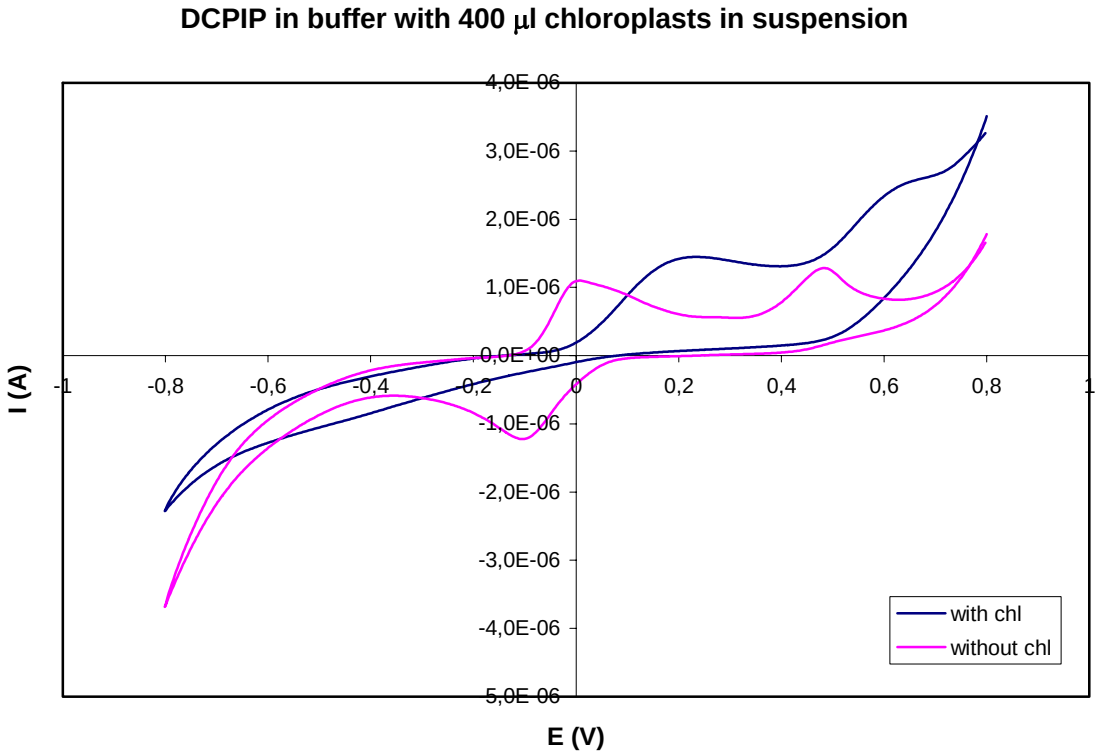


Figure 6.7: Cyclic voltammetry of DCPIP with and without chloroplasts in suspension. The measurements were made following 10 minutes of illumination with 10 W white light lamp.

In amperometric measurements the solution was illuminated for 4 to 5 seconds at 650 nm and 430 nm wavelengths every minute. The small signal (photocurrent) was expected from the illuminated electrode even in the absence of chloroplasts as it was shown in previous work on a platinum electrode (Carpentier *et al*., 1991). In the case of the carbon screen-printed electrode, used in our work, there was no response to light in the absence of chloroplasts. A solution containing only the buffer and the mediator was also tested in a control experiment (Figure 6.8) in order to demonstrate the photosynthetic origin of the photocurrent. The clear difference was observed in the experiments without (Figure 6.8) and with chloroplasts (Figure 6.9).

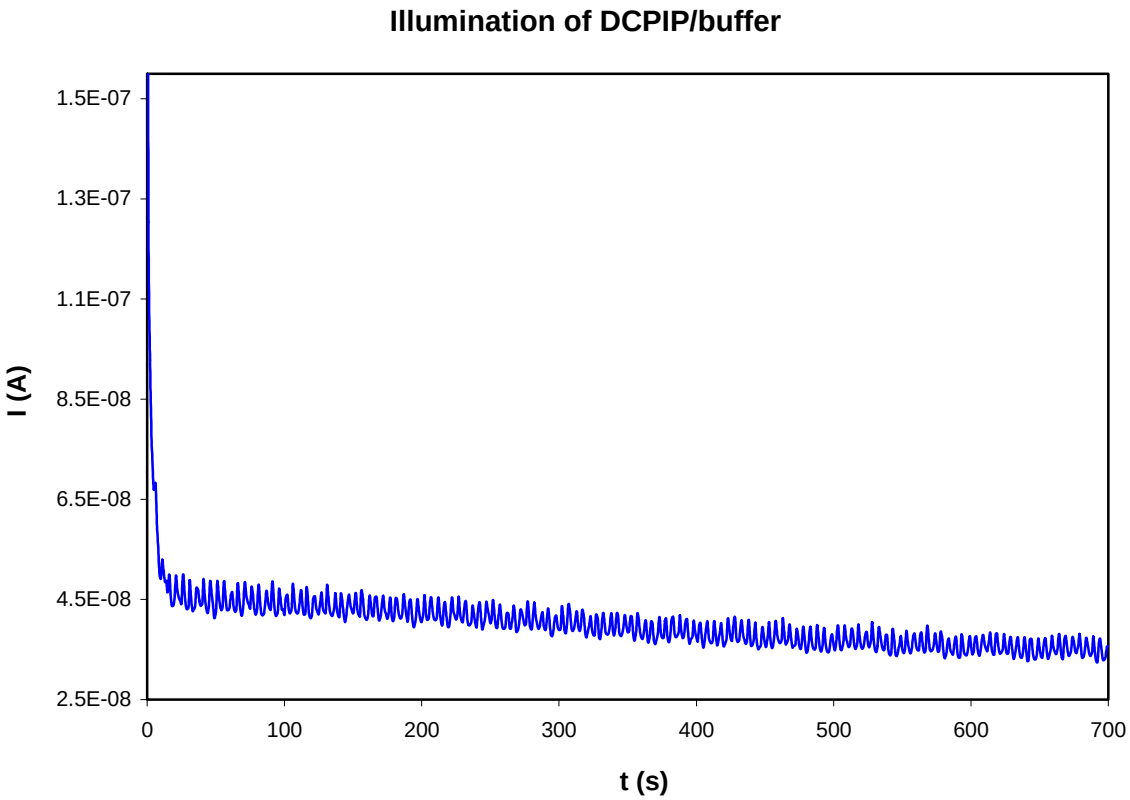


Figure 6.8: Illumination of buffer and mediator for a period of 700 seconds.

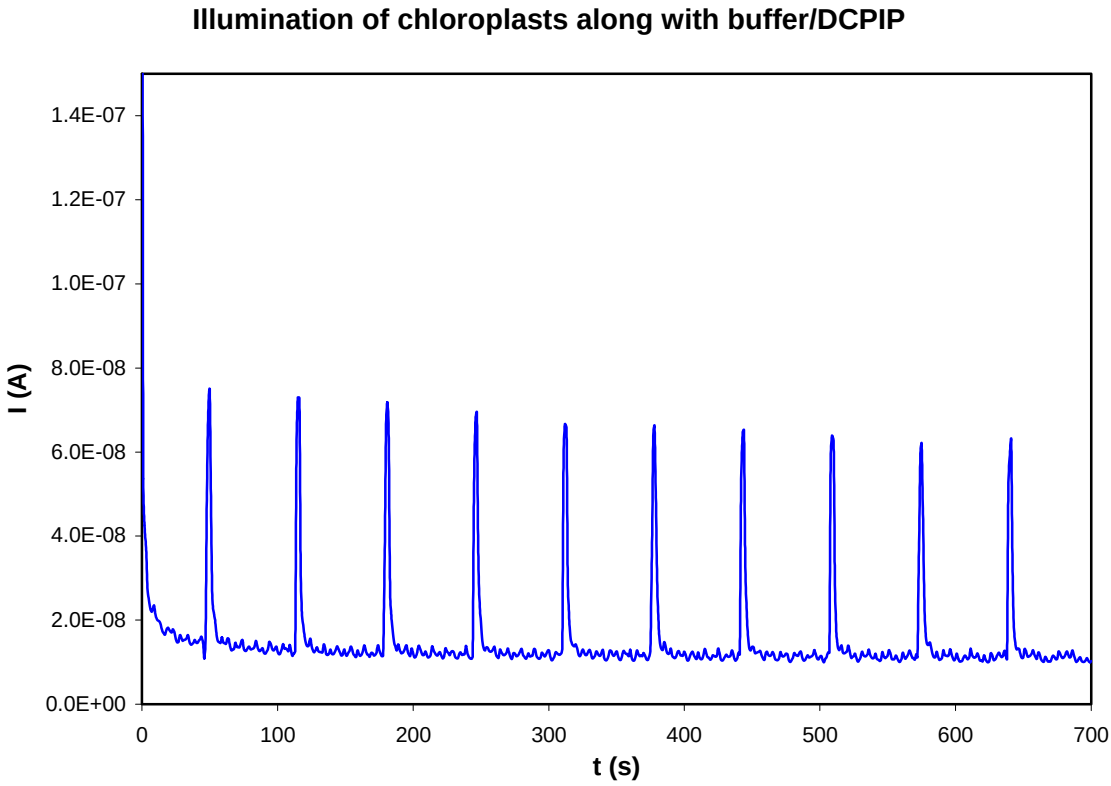


Figure 6.9: Illumination of chloroplasts in buffer and mediator for a period of 700 seconds.

Table 6.2: Chloroplast suspension response to continuous flashes.

Flashes (4-5 secs)	Current difference (ΔI) Amp
1st	5.91E-08
2nd	6.03E-08
3rd	6.03E-08
4th	5.82E-08
5th	5.36E-08
6th	5.50E-08
7th	5.33E-08
8th	5.16E-08
9th	5.08E-08
10th	5.22E-08

Figure 6.9 shows a typical signal from illuminated chloroplasts. The chloroplast suspension was continually fed into the cell and therefore the data shown here are not influenced by photoinhibition but only by the temperature related degradation of chloroplasts. The result for consequent measurements is shown in Table 6.2. There is a slight variation at each flash point which could be attributed to the difference in chloroplast suspension material available at the working electrode surface each time.

As expected the signals generated by thylakoids at 430 nm and 650 nm wavelengths were different (see Figure 6.10 and 6.11). The signal was larger at 650 nm which correlates with better energy conversion at this wavelength. There is a slight degradation of the signal with time that could be attributed to gradual loss of chloroplast activity. Furthermore, there is significant noise which is a result of the heterogeneity of the chloroplast suspension accessing working electrode surface. At both wavelengths the signal value was sufficient to be used in sensors.

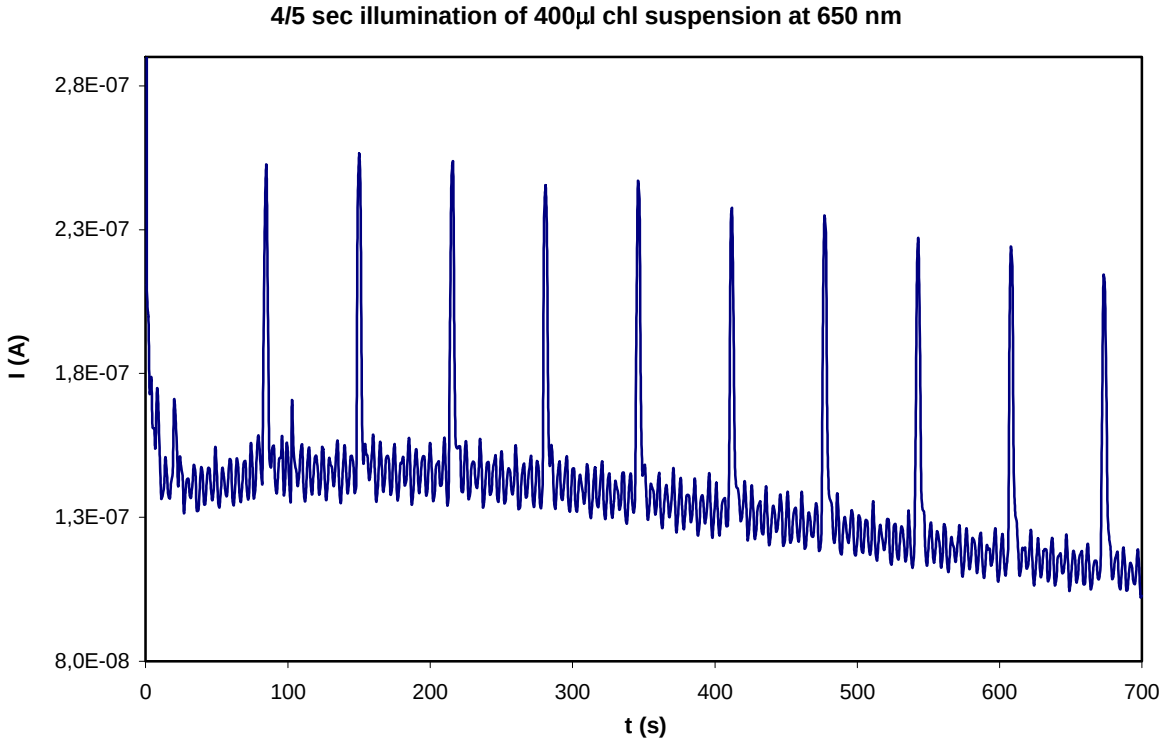


Figure 6.10: Illumination of chloroplasts at the wavelength of 650 nm.

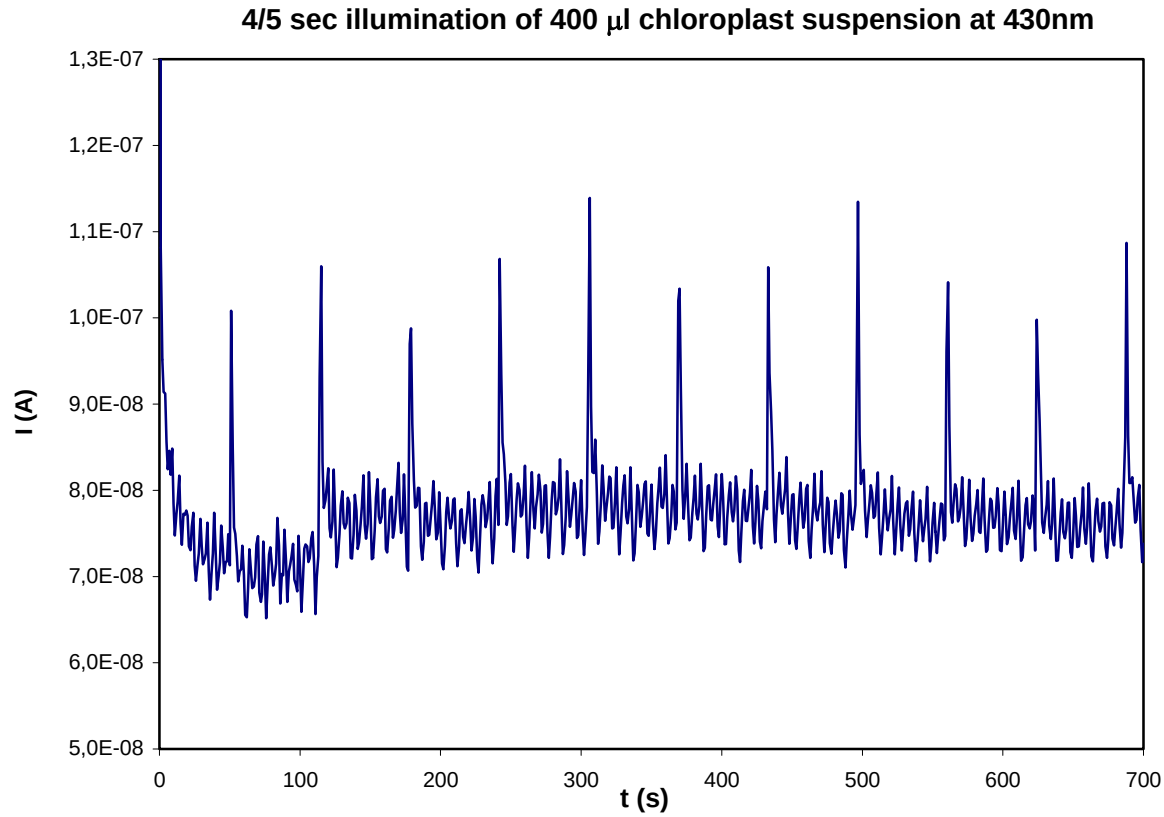


Figure 6.11: Illumination of chloroplasts at the wavelength of 430 nm.

6.3.4 Choice of wavelength

As has been discussed before, the absorption of light at both wavelengths varies considerably. To compare the sensitivity of chloroplasts to herbicide the measurements were repeated at these two wavelengths in the presence and in the absence of diuron. Diuron which is a photosystem inhibiting herbicide was added to the solution of chloroplast suspension and passed through the flow cell apparatus. Figures 6.12 and 6.13 show the decrease in the photocurrent produced at both wavelengths in response to 10^{-9}-10^{-6} M concentrations of diuron. After 100 seconds following addition of diuron a decrease in the signal at both wavelengths was observed. From the difference in current shown in Figures 6.12 and 6.13, calibrations curves were produced taking into account the error and the relative standard deviation of the data. From the calibration curves (Figure 6.14 for the 650 nm and Figure 6.15 for the 430 nm), the limit of detection was calculated for diuron additions.

The results at 650 nm differed from the results at 430 nm. At the 430 nm wavelength the electrochemical signal and the sensitivity were lower (μA), as expected, compared to the signal and sensitivity at 650 nm. The detection limit for diuron was 6.6 nM at 650 nm and 10.4 nM at 430 nm.

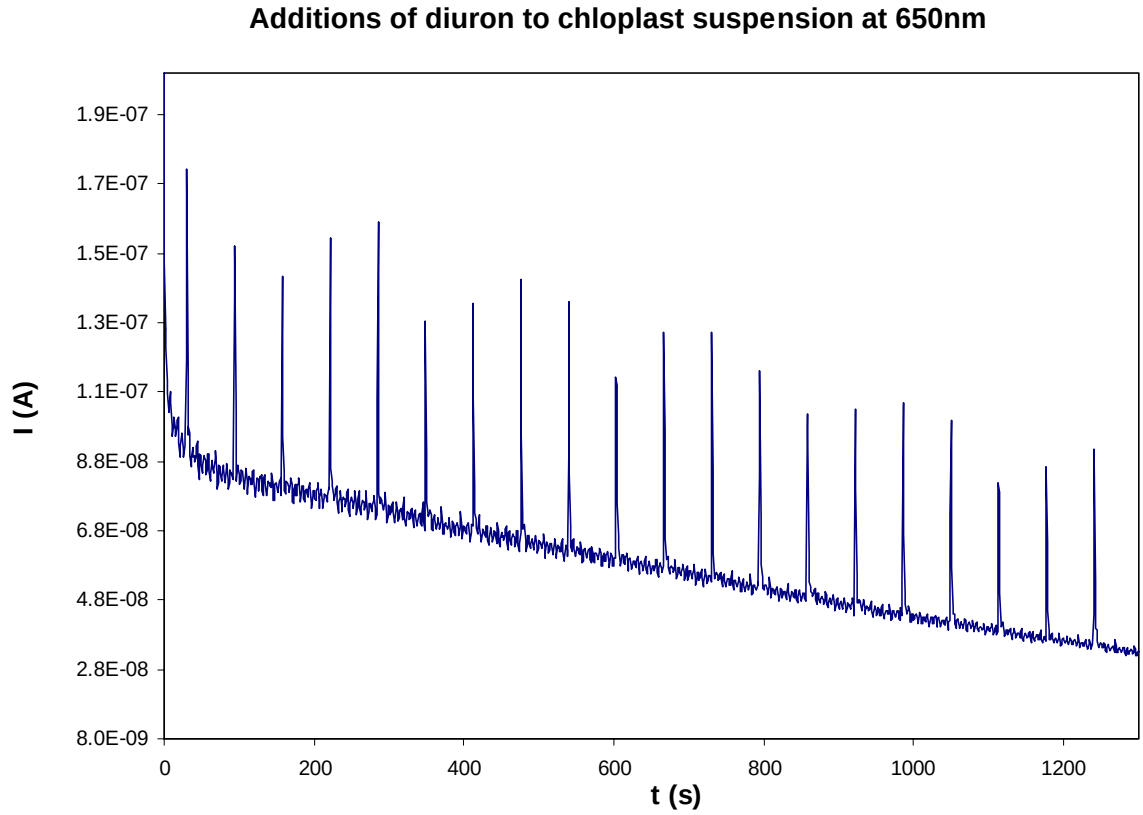


Figure 6.12: Illumination of chloroplasts at the wavelength of 650 nm with diuron inhibition.

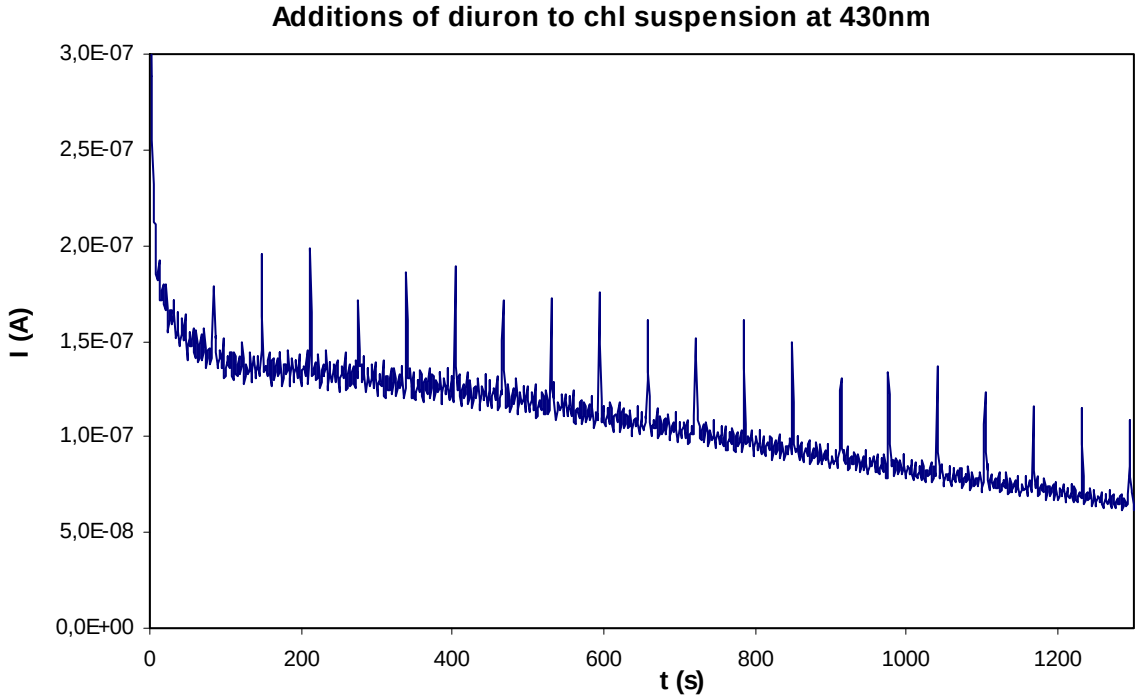


Figure 6.13: Illumination of chloroplasts at the wavelength of 650 nm with diuron inhibition.

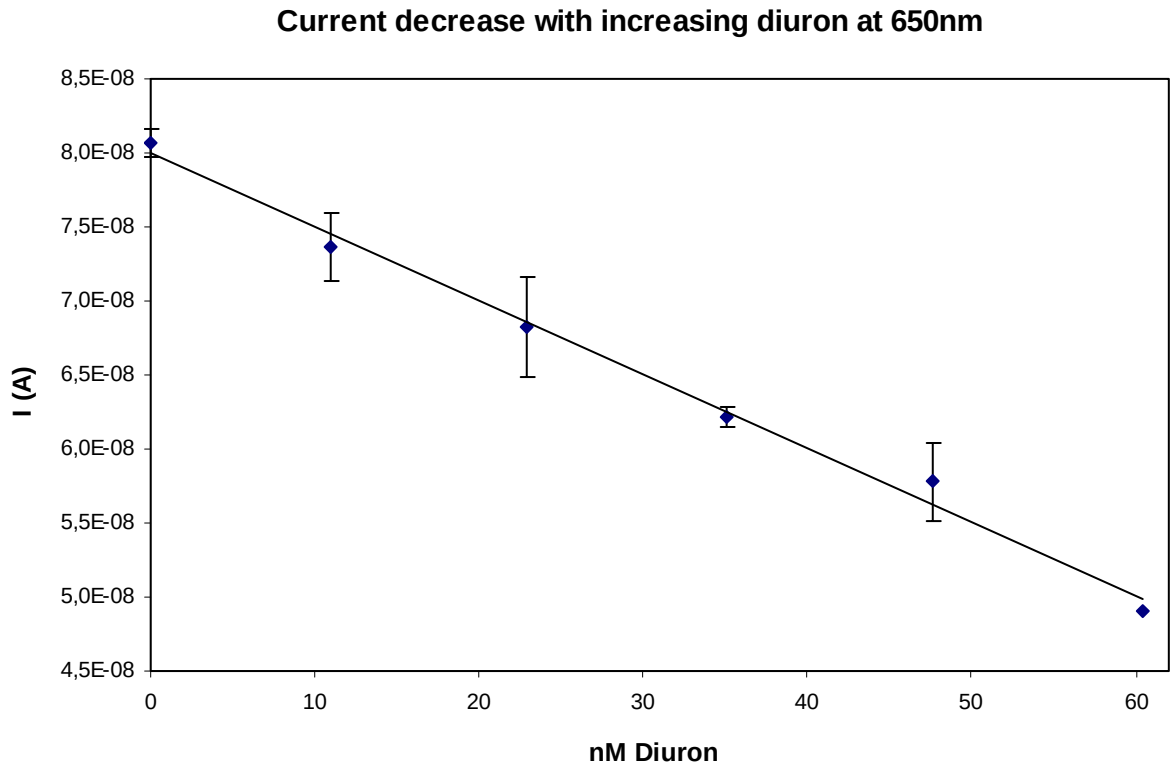


Figure 6.14: Calibration of diuron inhibition at 650 nm.

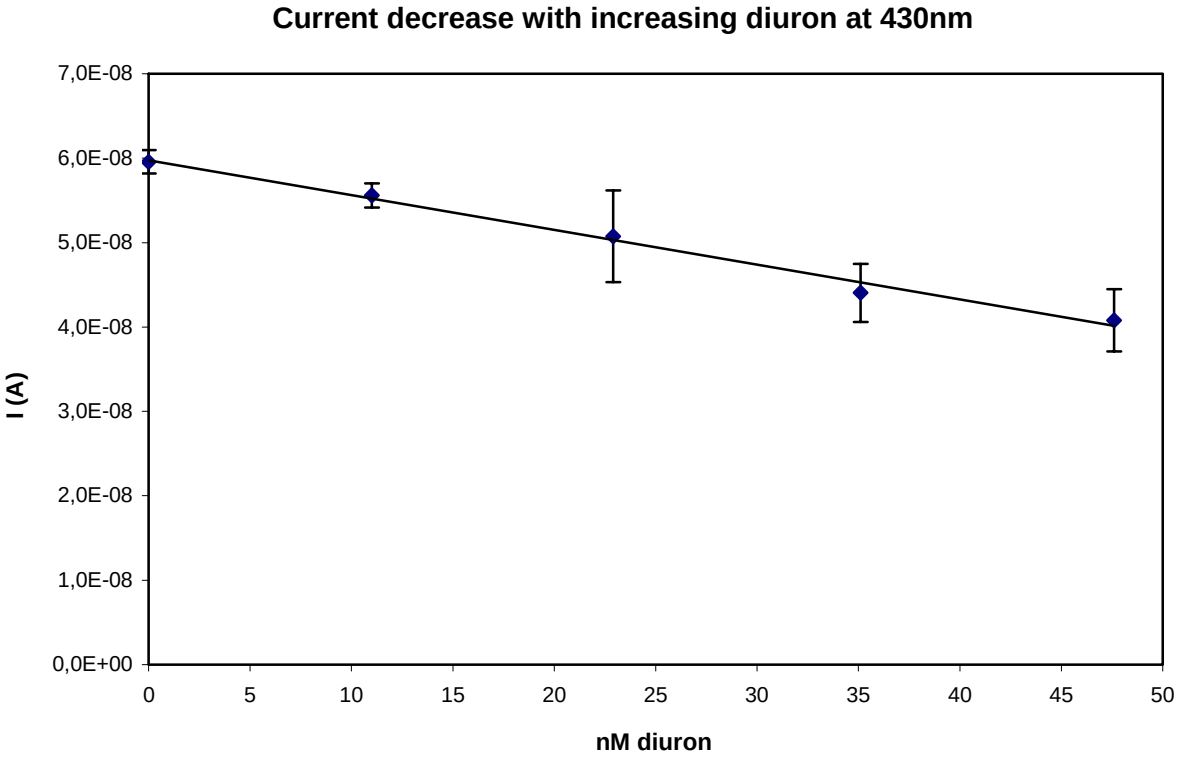


Figure 6.15: Calibration of diuron inhibition at 650 nm.

6.4 CONCLUSION

The testing of illuminated chloroplast proved that the amount of H_2O_2 generated by the system is not sufficiently high to be detected by the developed hydrogen peroxide sensor. The new protocol for the detection of herbicides involved a modified Hill reaction where the mediator – DCPIP is detected by chronoamperometry at potential 200 mV. Results from the flow injection analysis suggested that a sensitive detection of herbicide can be achieved using developed photoelectrochemical cell. The wavelength of 650 nm was optimal for the generation of photocurrent and for herbicide detection. In order to minimise the noise and to increase the sensitivity of measurements an immobilisation protocol was applied as shown in the following chapter.

CHAPTER 7: BIOSENSOR FOR HERBICIDES MONITORING

7.1 INTRODUCTION

Concerns over the pollution risk to drinking water from industrial or agricultural applications, has been rising and the need for continuous monitoring is recognised. This monitoring has to be rapid, easy to operate, and have low-cost screening procedures which could work in the field. In this chapter whole chloroplasts and thylakoids have been compared according to their performance in photoelectrical measurements previously described. The process of the Hill reaction, which was introduced in the previous Chapter, has been used for herbicide detection and two more mediators were also tested in the system. These include potassium ferricyanide (FeCy) and duroquinone (DQ). Several herbicides have been tested in this work using SPCEs and the response of the inhibited photocurrent is attributed to the relevant herbicide concentration.

In general, toxic chemicals wield their effect by interacting with biological systems at a cellular or biomolecular level (Wenzel *et al*., 1997). The photosynthetic process is largely similar for cyanobacteria and vascular plants. The light reaction of photosynthesis is the primary target of one half of herbicides used in agriculture. Binding of herbicides could be detected indirectly as an effect of the growth or the metabolism of the organism, but this approach would be too slow for use in screening applications. Recently direct methods have been developed for demonstrating the inhibition of photosynthesis by herbicides. These techniques are based on the fluorescence, oxygen evolution, and redox chemistry of the reaction centres of the photosynthetic pigments (Brewster, 1993). Electrochemical cells also have been used to monitor the transformation of absorbed light energy through the electron transport chain (Goetze and Carpentier, 1990; Agostiano *et al*., 1992; Brewster *et al*., 1995). This electron transport would require the synchronised activities of both photosystems for the production of a photocurrent. This enables the detection of herbicides acting at any location along the electron transport chain. A number of herbicides have being found to bind with high affinity to the reaction centre of photosystem II. In consequence, herbicides block the electron transfer from the excited state and prevent the reduction of the redox mediator. A simplified scheme for the photoreduction catalysed by photosystem II is shown in Figure 7.1. The scheme represents the PSII reaction under illumination conditions and under conditions used in

this work where the plastoquinone pool is replaced by a redox mediator such as DCPIP, FeCy and DQ.

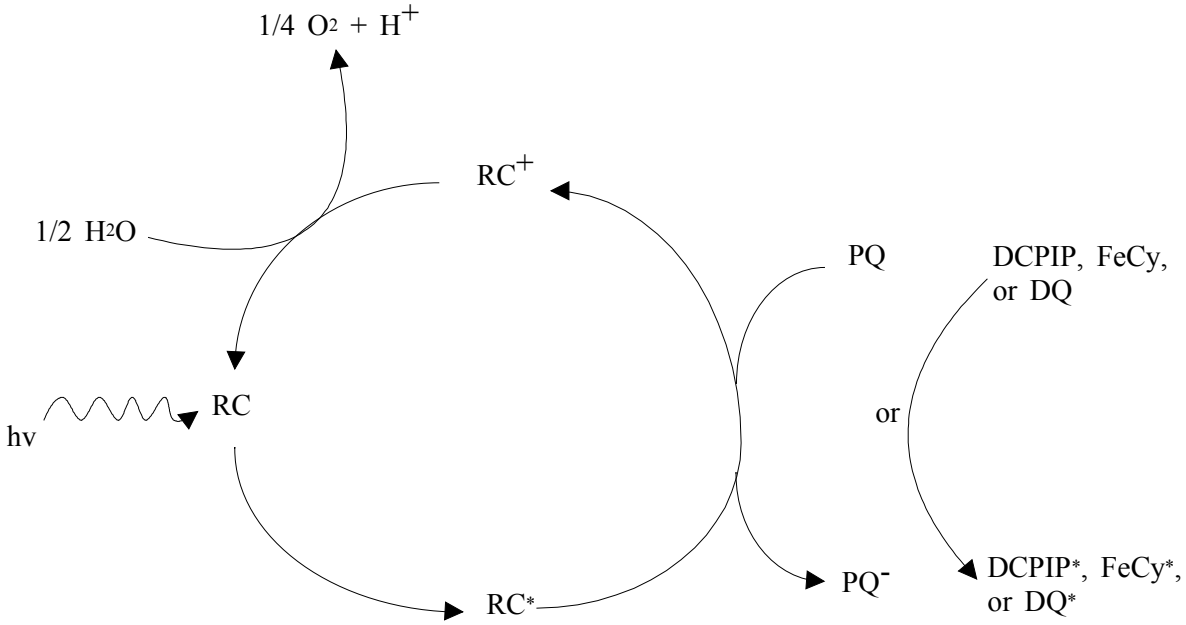


Figure 7.1: Simplified reaction scheme for the reaction system of photosystem II where the plastoquinone pool could be replaced by an artificial electron acceptor such as DCPIP, FeCy, and DQ.

In the scheme, RC is the reaction centre, PQ and PQ^- is the plastoquinone and its reduced form respectively, DCPIP and $DCPIP^*$ are the dichlorophenol indophenol and its reduced form respectively, FeCy and FeCy* are the potassium ferricyanide and its reduced form respectively, and DQ and DQ^* are the duroquinone and its reduced form respectively. The light energy is transferred to the P_{680} reaction centre of photosytem II, creating an excited state electron. Electron transfer on the acceptor side of PSII involves two quinones, QA and QB. The excited electron is passed to pheophytin and then to a protein-bound plastoquinone molecule called Q_A. Q_A, in turn, passes the electron to a protein-bound plastoquinone called Q_B via a nonheme iron. The hercibicides especially triazines (e.g. atrazine and simazine) and phenylureas (e.g. diuron) effect mainly the D1 protein of the reaction centre (Trebst and Draber, 1979). These herbicides inhibit photosynthesis by binding to the D1 protein at the binding site for Q_B. The binding

affinity of the herbicide greatly depends on the amino acid composition of the hydrophilic loop in the D1 protein (Piletskaya *et al*., 1999). The reacting herbicides block electron flow from the $Q_A^{\cdot -}$ (Q_A radical) to the Q_B pocket and stop photosynthesis. To develop a herbicide sensor we exploited inhibition of the photosynthetic process by pollutant using as a marker reduction of a synthetic mediator DCPIP.

In the past materials such as whole photosynthetic organisms, chloroplasts, thylakoids and extracted PSII particles have been used as biological material for sensors. Most work has been done on whole cells from spinach chloroplasts or thylakoids in biosensors (Cocquempot *et al*., 1981; Thomasset *et al*., 1983; Thomasset *et al*., 1986; Carpentier and Lemieux, 1987; Carpentier *et al*., 1989; Purcell *et al*., 1990; Carpentier *et al*., 1991; Loranger *et al*., 1994; Piletskaya *et al*., 1999; Koblitzek *et al*., 2002). The extraction of PSII particles is complicated and time consuming and therefore not enough work has been done with these fractions used as the photosynthetic material (Carpentier and Lemieux, 1987; Rouillon *et al*., 2000; Koblitzek *et al*., 2002). The immobilisation of intact cells from photosynthetic bacteria is a straightforward technique and several publications have been reported in the past from cyanobacteria (especially *Synechococcus elongates*) (Preus and Hall, 1995; Rouillon *et al*., 1999) and microalgae (Rawson *et al*., 1989; Frense *et al*., 1998; Sanders *et al*., 2001). The disadvantages of immobilisation of intact cells lie in the lack of permeability of the cell network to electrolytes and the possibility of losing the reactants and products through the side reactions of the cells. The largest amount of work so far has been performed on immobilised chloroplasts involving intact chloroplasts, a mixture of chloroplasts and photosynthetic membranes and thylakoid membranes alone. Spinach has been considered to be the best higher plant for extraction of chloroplasts and thylakoids since it is highly accessible, easy to handle and has large chloroplasts. From the chloroplast isolation, thylakoids could be prepared by osmotic shock in a hypotonic medium.

Different parameters and properties have been tested for evaluating the immobilisation procedure according to biological material, storage and operational stability. Immobilisation methods are classified as physical or chemical procedures according to whether or not they form covalent bonds (Papageorgiou, 1987). Physical methods usually refer to adsorption or gel inclusion for entrapping the biological material. Chemical immobilisation involves cross-linking with glutaraldehyde or glutaraldehyde

with different proteins such as gelatine, collagen, or bovine serum albumin. Covalent immobilisation on a solid support is the most common technique for enzyme immobilisation. In the case of photosynthetic material, the denaturing effect of the binding agent makes this technique unsuitable. However, glutaraldehyde has been shown to preserve the activity of the Hill reaction in chloroplasts which gives the possibility to be used as a chemical method for the immobilisation of photosynthetic material (Park *et al*., 1966). The denaturation could be minimised by the addition of a protein such as bovine serum albumin (Cocquempot *et al*., 1981; Thomasset *et al*., 1983; Thomasset *et al*., 1986; Carpentier and Lemieux, 1987; Carpentier *et al*., 1989; Purcell *et al*., 1990; Carpentier *et al*., 1991; Loranger *et al*., 1994; Piletskaya *et al*., 1999; Koblitzek *et al*., 2002). There is a covalent bond formed between glutaraldehyde and the amino acids proteins of PSII, in such a way that they form a polymer network, which allows the protection of the photosynthetic material against other possible binding material.

This work describes the development of a new herbicide sensor based on immobilised chloroplasts and a detection principle which utilises electrochemical version of Hill reaction. In this study chloroplasts were immobilised in a BSA-glutaraldehyde cross-linking matrix. In this type of immobilisation, glutaraldehyde creates covalent bonds with all species and more importantly with NH_2 free groups on both chloroplasts and albumin which helps to protect the chloroplasts from denaturing conditions. The screen-printed electrodes used for the immobilised material along with amperometric detection provided an easy and sensitive detection system.

7.2 MATERIALS AND METHODS

7.2.1 Reagents

Phosphate buffer for cyclic voltammetry, was made using Na_2HPO_4 and NaH_2PO_4 (BDH Laboratory Supplies, Merk Eurolab) and KCl purchased from Sigma. The chemicals used in chrono-amperometrical measurements were tricine (Sigma), sucrose (Fisher Scientific), sodium chlroride (NaCl) (Sigma), magnesium chloride ($MgCl_2$) (Sigma) and chloroamphenicol (CAP) (Sigma). The mediators used in cyclic voltammetry were potassium ferricyanide (FeCy) (Sigma), duroquinone (DQ) (Acros organics) and 2,6-dichlorophenol indophenol (DCPIP) (Sigma). The herbicides detected through

chrono-amperometry were atrazine diuron, simazine and diethylterbuthylazine purchased from Sigma. Sorbitol, MES (2(N-morpholino)ethane sulphonic acid) and Tris-Base were purchased from Sigma.

7.2.2 Safety considerations

Herbicides used in this work were all of analytical grade type Pestanal®. All herbicides used were dissolved in a methanol solution. With these compounds, it is better to avoid skin contact but in case of an accident it is necessary to rinse the skin with water and seek medical advice. Gloves were worn all time for handling herbicide solutions and preparations were executed in the chemical hood. Safety information for duroquinone revealed that toxicological properties of this material have not been investigated. In cases of skin contact it is advisable to remove all clothing and wash thoroughly with water. In case of breathing problems and limited fresh air, it is better to give artificial respiration and if this is not enough, give oxygen.

7.2.3 Chloroplasts and thylakoids preparation

Two protocols have been tested in this work. Chloroplast extraction was performed using the modified protocol described previously (Piletskaya *et al*., 1999): chloroplasts were isolated from fresh spinach leaves (*Spinacea oleracea L.)*. 100 g of leaves were washed with water, dried on filter paper and homogenised in 300 ml of extraction buffer containing 0.35 M sucrose, 50 mM Tris-HCl buffer, pH 8.0 and 10 mM NaCl. The homogenate was filtered through 4 layers of cheese-cloth and through sieves with a pore diameter of 100 μm. Centrifugation was performed for 10 min at 2 000 g (Model J2-21 centrifuge Beckman, USA). The pellet was re-suspended in the extraction buffer containing 1% of BSA. Aliquots (200-μl) were placed in Eppendorf tubes and kept at –80 ^{0}C. Chlorophyll concentration was measured and adjusted to 3 mg/ml.

7.2.4 Electrochemical analysis

The techniques used for the analysis and evaluation of the analytes were cyclic voltammetry and chrono-amperometry. All measurements were performed with an Autolab Electrochemical Analyser (μ-autolab) with the general purpose electrochemical

software GPES 4 (Ecochemie, Utrecht, Netherlands). All potentials are referred to a silver/silver chloride screen-printed electrode. Data from both electrochemical applications were processed through a spreadsheet software package, Microsoft Excel, in order to produce relevant graphs and measurements. Screen printed electrodes were connected to an IDE edge connector from Maplin Electronics plc (Wombwell, South Yorkshire, UK) and they were insulated at one side for a tighter connection. The multimeter flow cell system (Das Srl, Italy) was used for all chrono-amperometric measurements at the wavelength of 650 nm.

For cyclic voltammetry measurements the buffer solution used was a phosphate buffer at a concentration of 40 mM containing 0.1 M KCl and 10 μl of chloroplast suspension. The voltammetric scan was cycling between -0.8 V to 0.8 V and the scan rate was set to 50 mV. All measurements were reproduced at least three times and all scans were performed at ambient temperature (25 ^{o}C) in the dark. Duroquinone starting concentration was 100 mM and it was dissolved in methanol. Potassium ferricyanide was prepared in the phosphate buffer at a starting concentration of 50 mM. Both mediators were detected tenfold in the buffer before carrying out CV.

Amperometric data were performed at the oxidation potential of DCPIP which was found to be 200 mV (Chapter 6). BSA (bovine serum albumin)/GA (glutaraldehyde) matrix was used for immobilisation of chloroplasts. The actual mixture consisted of 1.5% glutaraldehyde, 2% BSA, 40 mM phosphate buffer pH 7.4, and 3 mg/ml of chloroplasts. From this mixture, a quantity of 5 μl was deposited onto the electrode surface and left for approximately one day or more at -80 ^{0}C, -25 ^{0}C and 4 ^{0}C. To calculate the chlorophyll content a previous protocol was used (Ferus and Arkosiova, 2001). The equation used is as shown:

$$\text{Chl (a+b) (mg/l)} = (7.15 * A_{663} + 18.71 * A_{647}) * D \qquad (7.1)$$

where A is the absorption at the given wavelengths and D is the thickness of the cuvette (1 cm).

The half life of the immobilised material was determined by illuminating it for 12 hours. Illumination pulses in chronoamperometry lasted for 5 seconds and they are repeated

every 10 minutes in order to avoid any effect of photo-inhibition in the photoproduction from the chloroplasts. The buffer for chronoamperometric measurements contained 20 mM Tricine, 70 mM sucrose, 15 mM NaCl, 5 mM $MgCl_2$ and 5 x 10^{-5} M CAP at a pH of 7.8. The final set up for herbicide measurements with the amperometric biosensor is shown in figure 7.2.

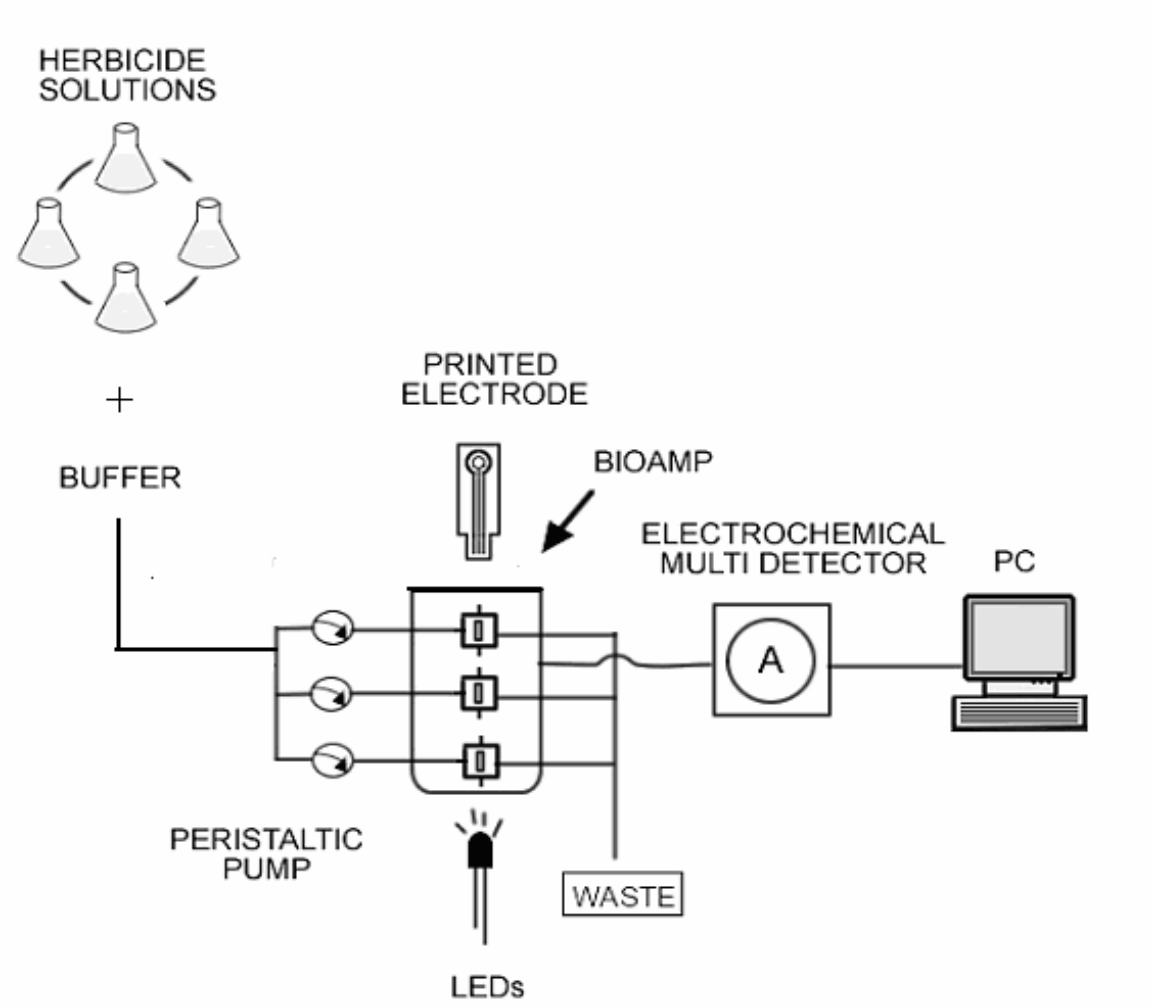


Figure 7.2: Final set for multibiosensor herbicide analysis.

River water was analysed for the total concentration of herbicides present in the water. The water was collected on April 2004 by Pontelagoscuro (Ferrara, Italy) from the River Po. The Po water was filtered through a pre-filter (Glasfaser Rundfilter, 142mm,

Schleicher & Schuell, Germany) and a 0.45 μm filter (nitrate cellulose, 142 mm, Sartorius AG, Germany). The filtered water was stored at 4^{o}C into 10 l glass bottle prior to analysis.

The herbicides in the water extract were analysed using the GC-MS\MS Ion-Trap technique. The gas chromatographic conditions were: PTV injection in a splitless mode; column Varian VF-5ms FS 60m x 0,25 mm ID x 0.25 um; injected volume: 2 μl; carrier gas: He 1 ml\min with constant flow; transfer line: 280 °C. The mass spectrometer conditions were: EI 70 eV; standard autotune; multipler 1075 V; positive polarity; AGC target 50; temperature source: 250 °C; damping gas He, constant flow 0.3 ml\min. Under these conditions a calibration curve was undertaken, using the precursor and the monitored ions reported in the results section, for the analysis of terbuthylazine, simazine, atrazine, DET, DEA, metolachlor, alachlor, oxadiazon, prometryn. The data were provided by the Water Research Institute (IRSA-CNR) in Milan, Italy.

7.3 RESULTS/DISCUSSION

7.3.1 Cyclic voltammetry of mediators

Apart from DCPIP which was tested under cyclic voltammetry and found to have an oxidation potential at + 200 mV, two more potential mediators of the Hill reaction were tested. Data were recorded for mediators in the dark or under illumination. From these results, the potential suitable for amperometry was chosen. The scans for FeCy and DQ are shown in Figure 7.3 and 7.4, respectively. The oxidation potential of duroquinone shifts from approximately 0 mV without the illuminated chloroplasts to 200 mV with illumination of the chloroplasts. Both potentials are sufficient to use in order to avoid any interference. Unfortunately the fact that duroquinone has relatively poor solubility does not make it an attractive mediator to use with living systems such as chloroplasts. In addition, duroquinone has been considered by many as a potential inhibitor of photosystem II (Garstka *et al*., 2004).

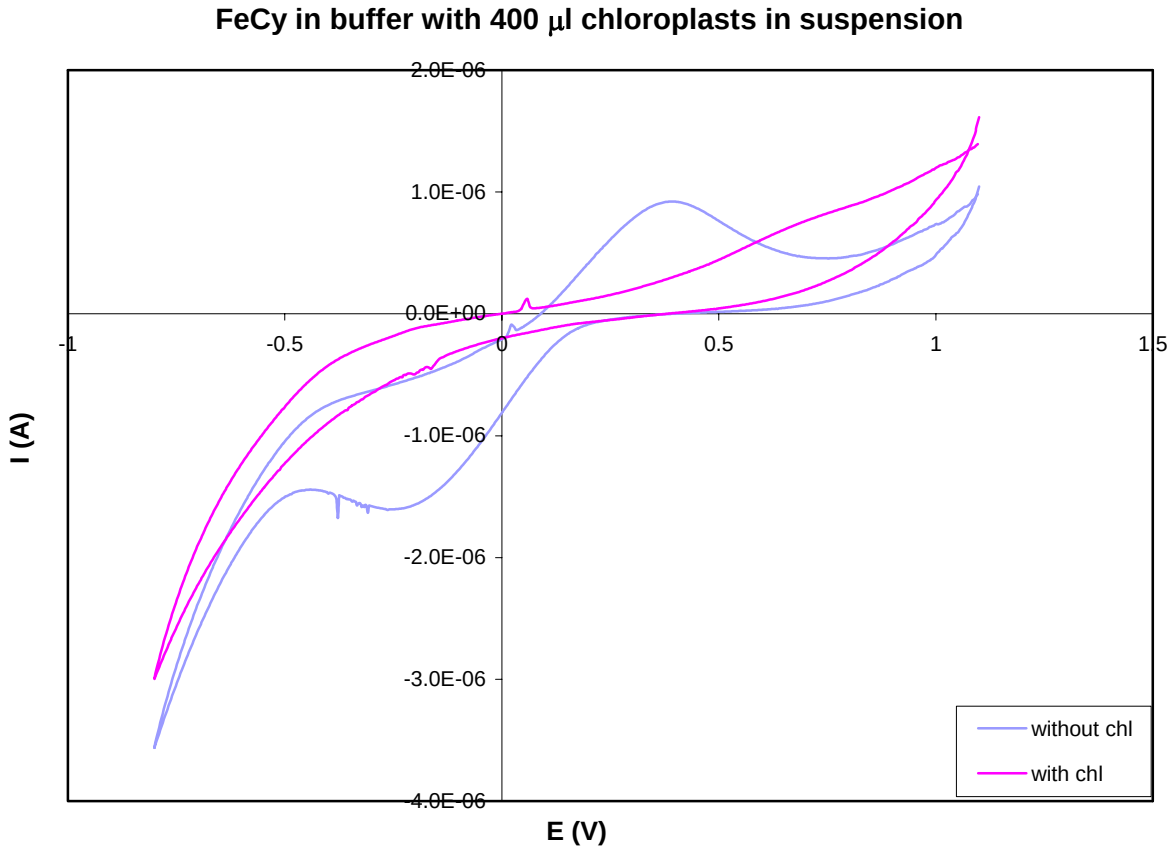


Figure 7.3: Cyclic voltammetry of FeCy with and without chloroplasts in suspension. The measurements were made following 10 minutes of illumination with a 10 W white light lamp.

For potassium ferricyanide scans without the chloroplast suspension the oxidation peak was detected at 450 mV and with the chloroplasts at 650 mV. Potassium ferricyanide was used as an acceptor for both photosystems (Ochiai *et al.*, 1982; Lemieux and Carpentier, 1988). In all cases the potential shift in the presence of chloroplasts under illumination has been observed and assigned to the change in movement of electrons in the thylakoid redox system.

Potassium ferricyanide mediators required a considerably high potential (overpotential) and are therefore not really suitable for use in environmental applications of the sensor. Therefore, DCPIP was considered a better mediator to use with a potential of 200 mV and no solubility problems. Replacement of the plastoquinone pool with each mediator provided a different redox potential suitable for use under amperometric

techniques. Since DCPIP had the optimum potential of 200 mV under illumination of chloroplasts (Chapter 6) it was chosen for the amperometric measurements with immobilised material on the electrode surface.

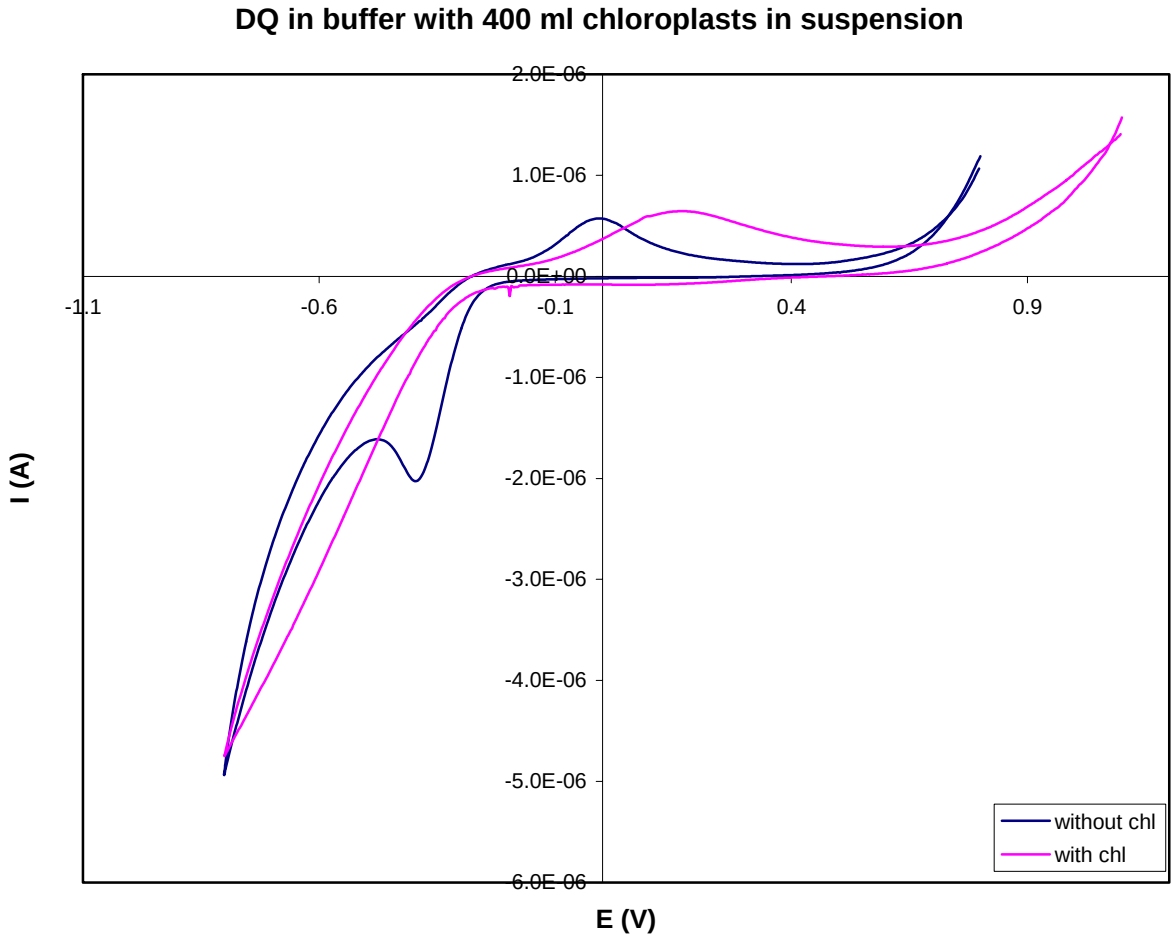


Figure 7.4: Cyclic voltammetry of DQ with and without chloroplasts in suspension. The measurements were made following 10 minutes of illumination with a 10 W white light lamp.

7.3.2 Immobilisation of chloroplasts

For the amperometric photosynthetic signal chloroplasts have been used. Materials were immobilised under the following conditions: 37 μl of 40 mM phosphate buffer pH 7.4, 28 μl of 2 % w/v BSA in phosphate buffer, 23 μl of GA 1.5 % w/v in phosphate buffer were mixed together and left for 5 minutes at room temperature. Then 14 μl of chloroplasts were added ([Chl] = 3 mg/ml) and both 5 μl and 10 μl of the total mixture were deposited on the screen-printed electrode surface. The electrodes were then left for at least 24 hours at $-80\ ^{0}C$. The excitation wavelength used for all measurements was 650 nm. The flashes were repeated every 10 minutes in order to allow full recovery and avoid any photoinhibition effect on the immobilised material. In the past, it was proven that prolonged strong light causes severe stress to the photosystem II complex.

After 24 hours immobilisation the signal from chloroplasts gave a reasonably high signal. The signal was found to be more stable and without the deviation observed with chloroplasts in suspension. The noise of measured signal was minimal. By adjusting the flow rate at the peristaltic pump, noise was minimised to a satisfactory level for calculating the current difference (ΔI). The optimal flow rate that was used for all measurements was 400 μl/min. The calculated signal from the illuminated chloroplasts was found to be in the area of 70 nA (Figure 7.5) for 5 μl deposited on the electrode surface and 90 nA for 10 μl (Figure 7.6). The chloroplasts signal seemed to have a steady plateau from the first illumination. An increase of immobilised material resulted in the increase of electrochemical signal.

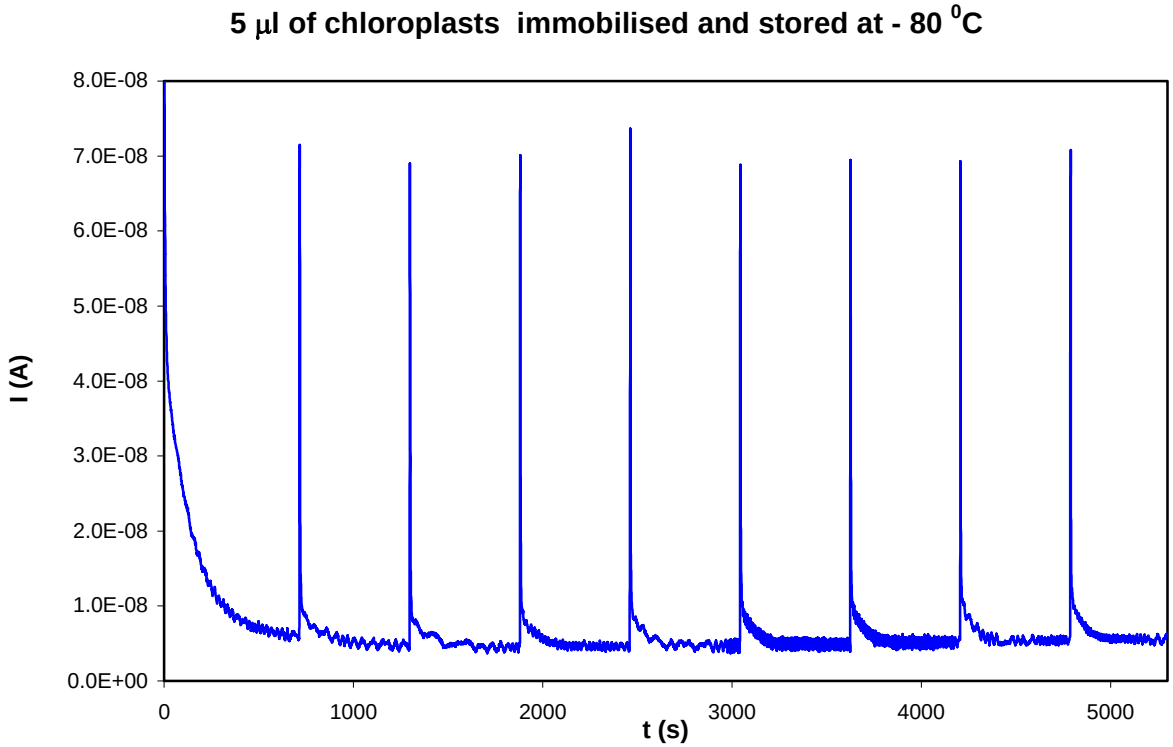


Figure 7.5: 5 µl of chloroplasts immobilised with GA/BSA and left overnight at – 80 ^{0}C.

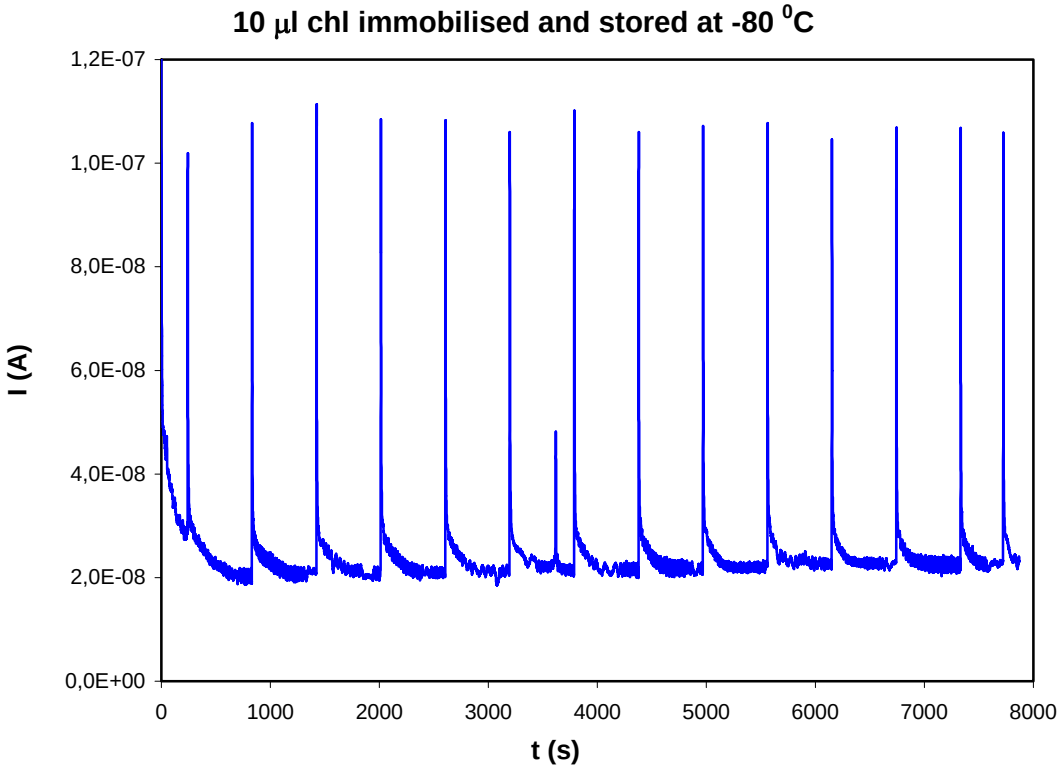


Figure 7.6: 10 µl of chloroplasts immobilised with GA/BSA and left overnight at – 80 ^{0}C.

Immobilisation using GA/BSA was efficient for continuous measurements for up to 3 hours. Bovine serum albumin has been proved in the past to stimulate markedly various photoreactions (Friedlander and Neumann, 1968). It has been proved that the action of BSA during the homogenisation step is to bind the endogenously released unsaturated fatty acids. Thus, it is known as the "suicide" protein (Friendlander and Neumann, 1968). In another work similar experiments were performed with thylakoids (Touloupakis *et al*., 2005). Apparently the thylakoids generated similar results as the chloroplasts. Efficiency of the produced photocurrent could be proved through the measurements of the relative standard deviation (equation 7.2). The RSD of the electrodes with chloroplasts was found to be 9.11 %.

RSD = (Average (I) / Standard deviation (I)) * 100 % (7.2)

7.3.3 Storage stability

The data presented here are under different storage conditions. Chloroplasts were immobilised in exactly the same way as described before, and an aliquot of 5 μl of chloroplasts was deposited on the electrode surface and electrodes were placed at 4 ^{0}C (Figure 7.7), -20 ^{0}C (Figure 7.8), and – 80 ^{0}C (Figure 7.9), in order to check the storage stability. The stability of the sensor is dependent on the storage (preservation) conditions to which the chloroplast have been subjected to. Furthermore, operational stability of the photosynthetic material has to be considered and taken into account. This was done by exposing the material to light and laboratory temperature conditions. Any change in room temperature affected the electrochemical signal and therefore all measurements were made under the same conditions (20 ^{0}C). Furthermore, immobilised chloroplasts have been kept under dark storage conditions at all temperatures, since previous work has demonstrated faster degradation rates in material stored in the light compared to the ones kept in the dark (Loranger and Carpentier, 1994).

All chloroplasts stored at different temperature generated signals with roughly similar amplitude. The chloroplasts immobilised at 4 ^{0}C generated a signal of low amplitude which increased with further illuminations (Figure 7.7). The signal decreased rapidly making measurements impossible after 1 hour. Nevertheless the storage temperature of 4 ^{0}C is good for measurements up to 1 hour but possibly only two different

concentrations of herbicides could be measured since they would require approximately 10 minutes for inhibit the signal from the chloroplasts. At – 20 ^{0}C, the signal lasted for longer but again a decrease in the current was registered indicating either rapid loss of material from the electrode surface or rapid deactivation of the chloroplasts (Figure 7.8). At the latter conditions a significant drop in the signal was observed after approximately 1 hour of continuous measurements. Storage at -80 ^{0}C was most efficient allowing continuous measurement for approximately 3 hours (Figure 7.9 and Table 7.1).

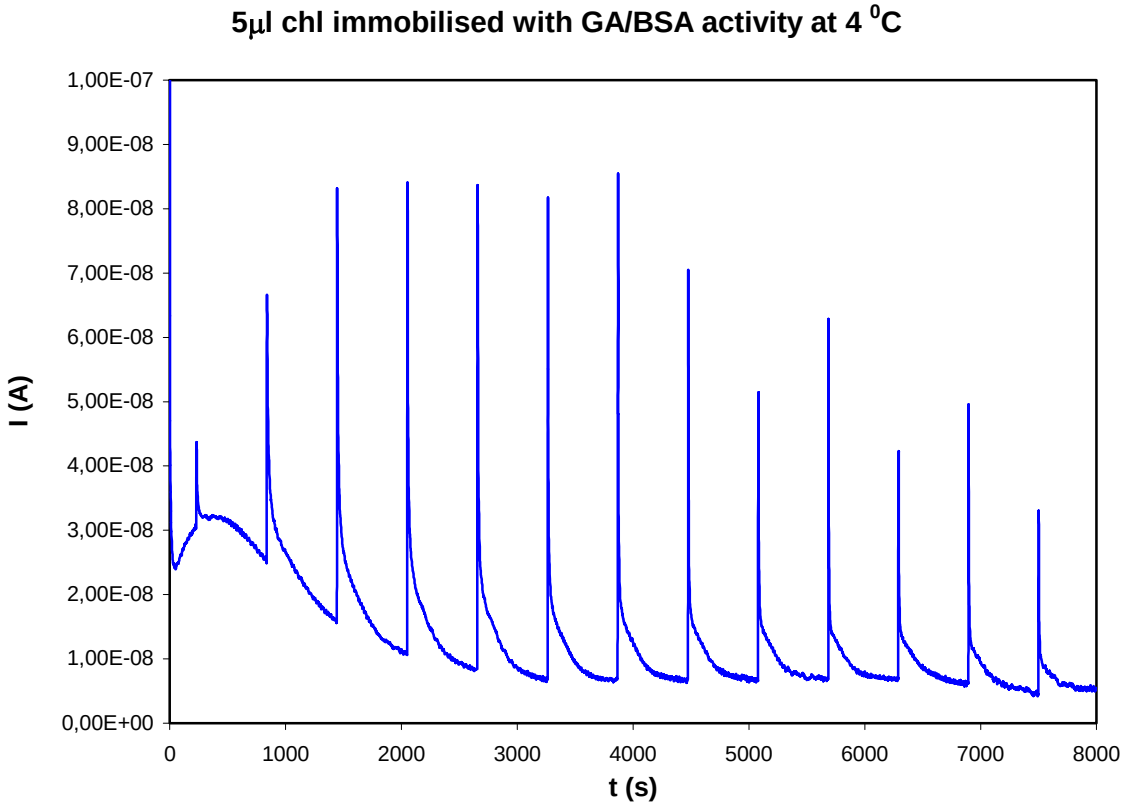


Figure 7.7: Performance of chloroplasts immobilised at 4 ^{0}C.

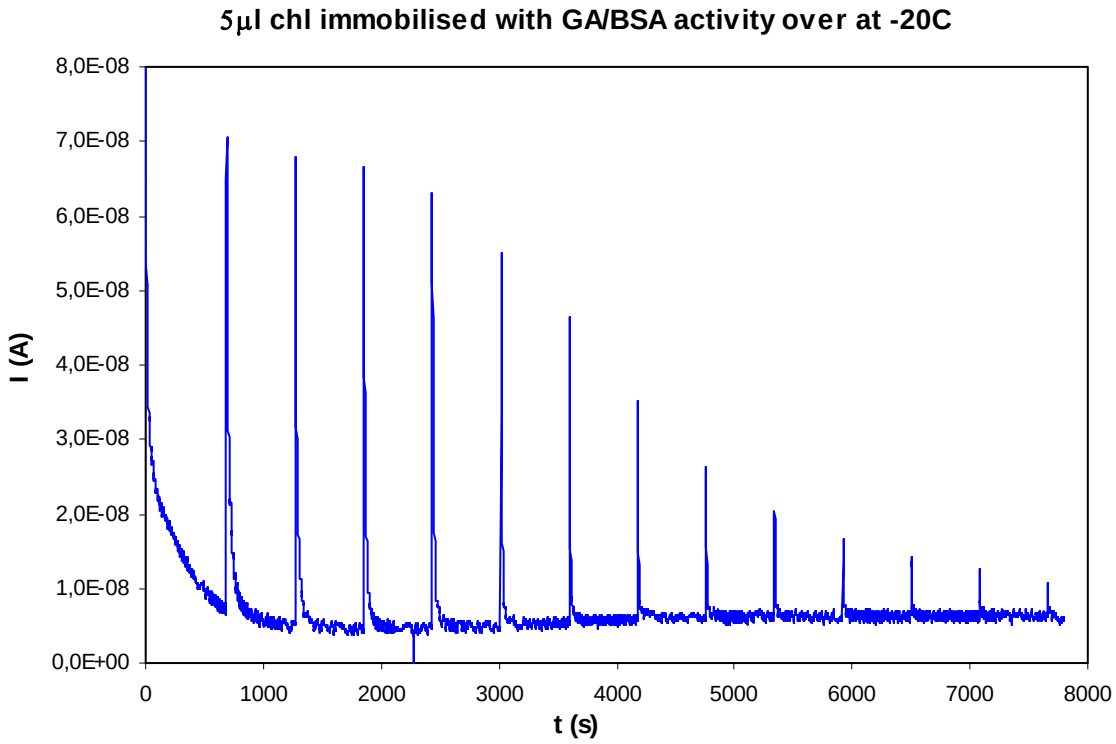


Figure 7.8: Performance of chloroplasts immobilised at -20 ^{0}C.

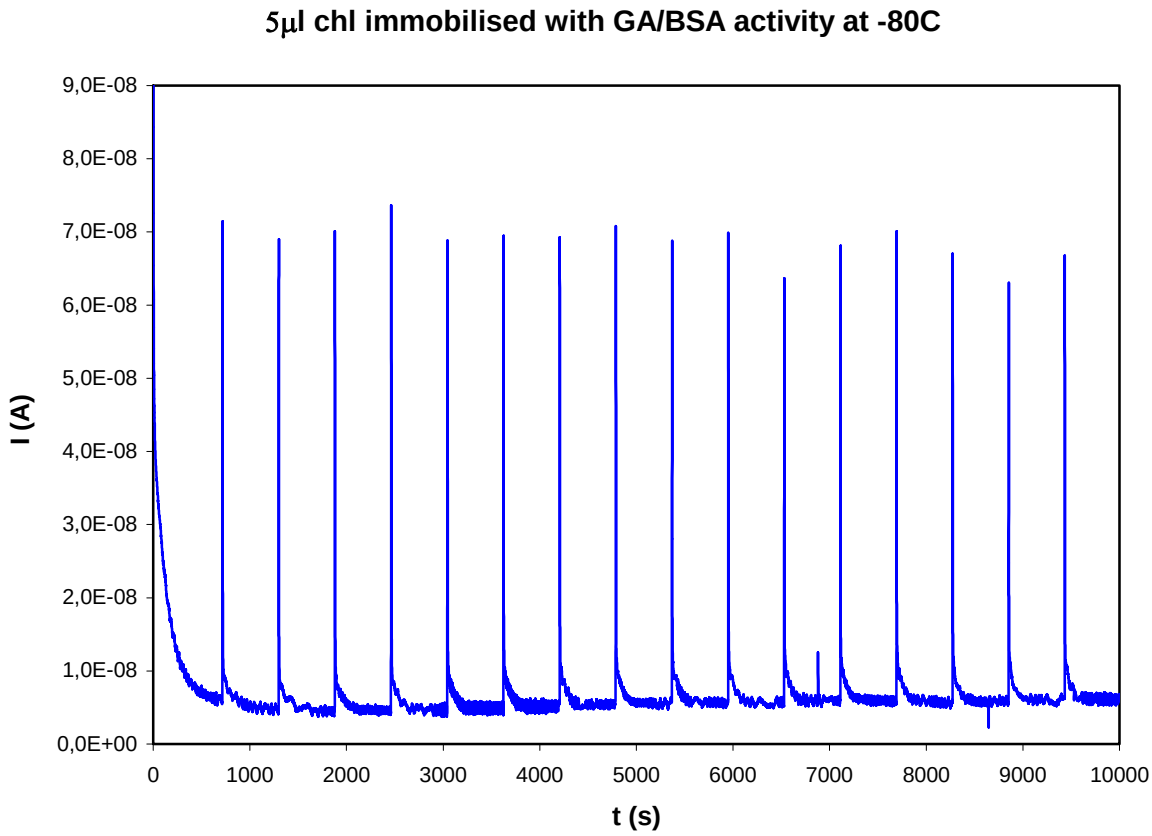


Figure 7.9: Performance of chloroplasts immobilised at -80 ^{0}C.

The signal resulting from chloroplasts under storage at – 80 ^{0}C for 1 day has been proven sufficient for herbicide measurements for up to 3 hours. The half life was found to last up to 12 hours of continuous measurements. In addition, electrodes with 10 μl immobilised material were kept for 3 months at this temperature and have shown a similar signal (Figure 7.10). The signal amplitude was again in the area of 105 nA – 95 nA and the relative standard deviation of the signal dropped to the value of 5.72 %. Furthermore the noise was minimised even more. Thus, the sensor for environmental measurements could be stored for at least 3 months under these conditions. Even thought the RSD levels for the signal were reasonably low, the RSD value between each electrode was found to be 10 %. This value is relatively high for electrochemical measurements but it could be improved by improving the fabrication procedure, making it more controlled, preferably by using automatic steps to exclude human error.

Table 7.1: 5 μl Chloroplasts signal after flashes at storage stability of – 80 ^{0}C.

Flashes (4-5 secs)	Current difference (ΔI) Amp	Flashes (4-5 secs)	Current difference (ΔI) Amp
1st	6,400E^{-08}	9th	6,223E^{-08}
2nd	6,454E^{-08}	10th	6,372E^{-08}
3rd	6,479E^{-08}	11th	5,729E^{-08}
4th	6,857E^{-08}	12th	6,150E^{-08}
5th	6,402E^{-08}	13th	6,436E^{-08}
6th	6,522E^{-08}	14th	6,138E^{-08}
7th	6,337E^{-08}	15th	5,721E^{-08}
8th	6,389E^{-08}	16th	6,0454E^{-08}

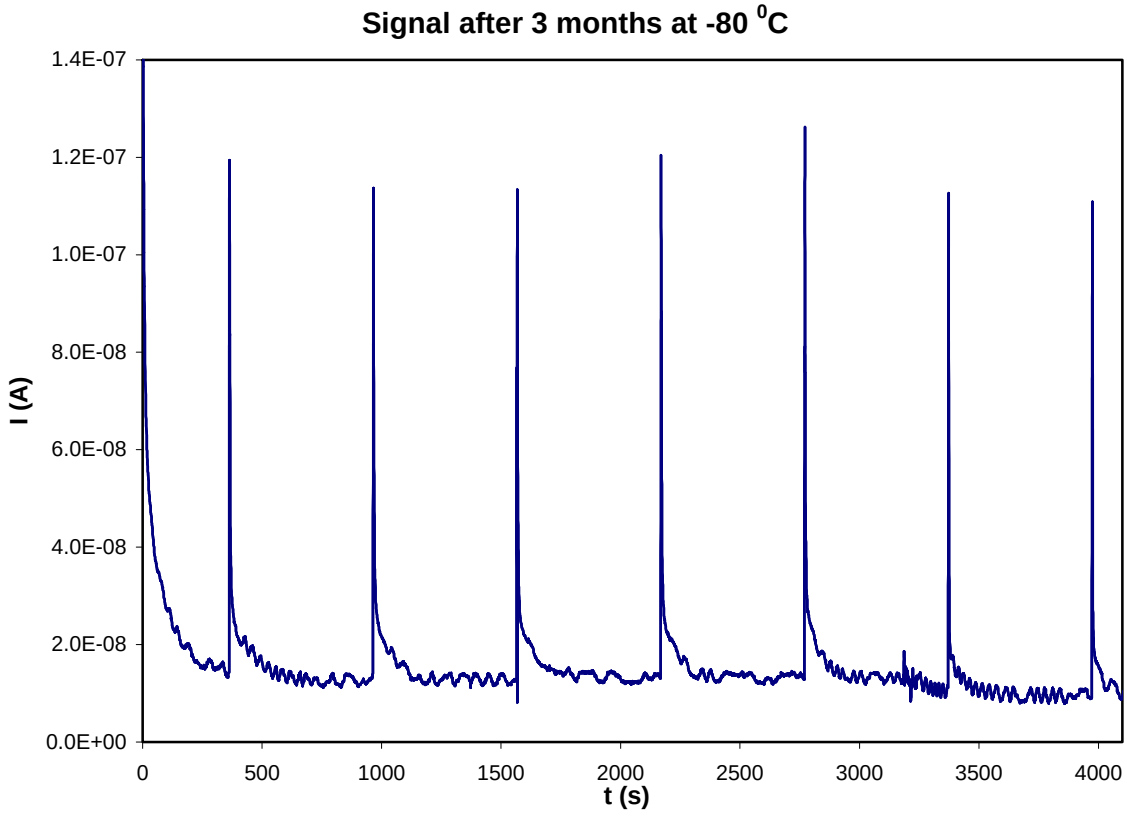


Figure 7.10: Operational stability for 3 months of 10 µl immobilised chloroplasts at storage temperature of – 80 ^{0}C.

7.3.4 Herbicide detection

Herbicide detection was performed under the same immobilisation conditions, with 10 µl on the electrode surface and the storage conditions of – 80 ^{0}C. Four herbicides, atrazine, simazine, deethylterbuthylazine and diuron were detected using the flow cell at 650 nm wavelength. A 10^{-4} M solution of each herbicide was prepared and from that several dilutions down to 10^{-8} M concentration. During measurements several additions of herbicides were made leading to a final concentration in the running buffer ranging from 1 x 10^{-9} M up to 1 x 10^{-6} M. The detection limit for each herbicide varied from 2.1 nM to 9.6 nM (Figure 7.11-7.14). Calibration curves for each herbicide were analysed using the Langmuir adsorption isotherm (Figure 7.15):

$$act = 100 - 100 \times [H]/ (I_{50} + [H]) \quad (7.3)$$

where *act* is the residual activity of the biosensor (in percent) after the addition of the herbicide, [H] is the concentration of the herbicide in the solution and I_{50} is the concentration causing the 50 % inhibition of the activity (equal to the RC-herbicide complex dissociation constant). The limit of detection was determined using the modified relationship for the Langmuir adsorption isotherm, (Koblitzek *et al*., 2002) as:

$$LOD = 2.6x\ RSD\ x\ I_{50} / (100 - 2.6\ x\ RSD) \quad (7.4)$$

The results are showed in Table 7.2. The data also showed a RSD varying from 2% to 7%. Each measurement was made in triplicate and the average data plotted together with relative standard deviation values (see e.g. Figure 7.11). The interval between each measurement was 10 minutes.

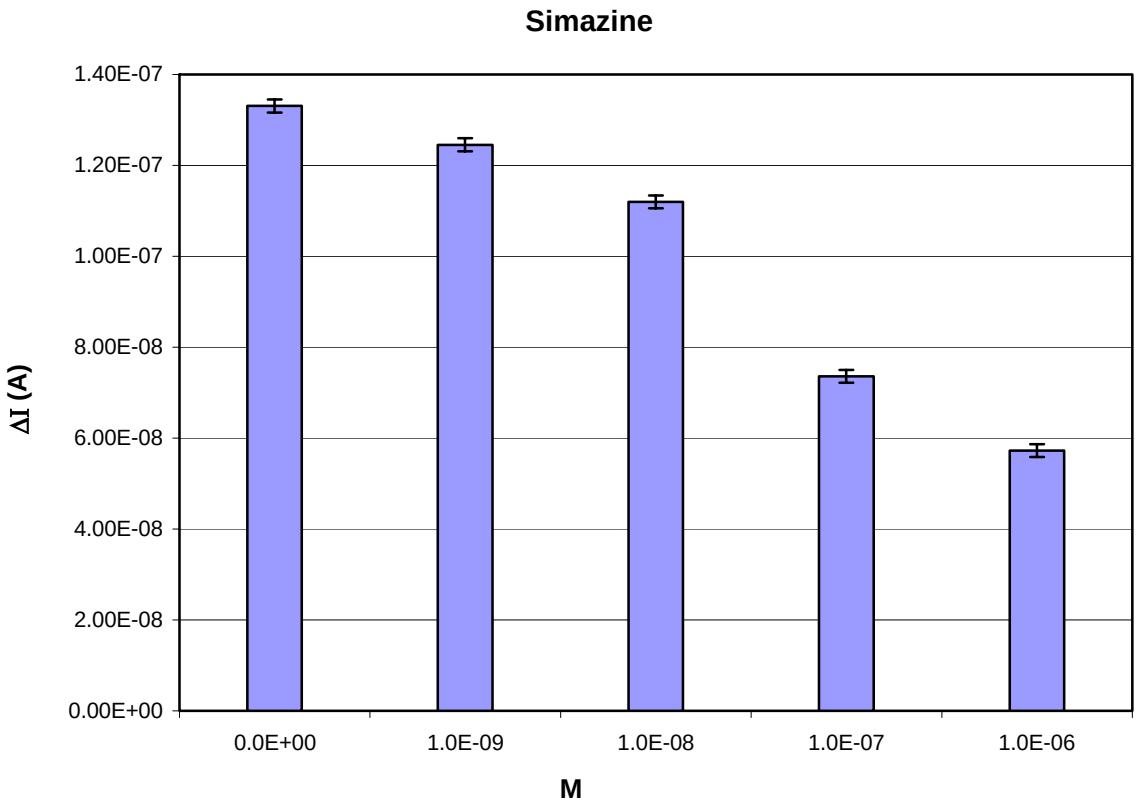


Figure 7.11: Sensor responses (ΔI) for simazine.

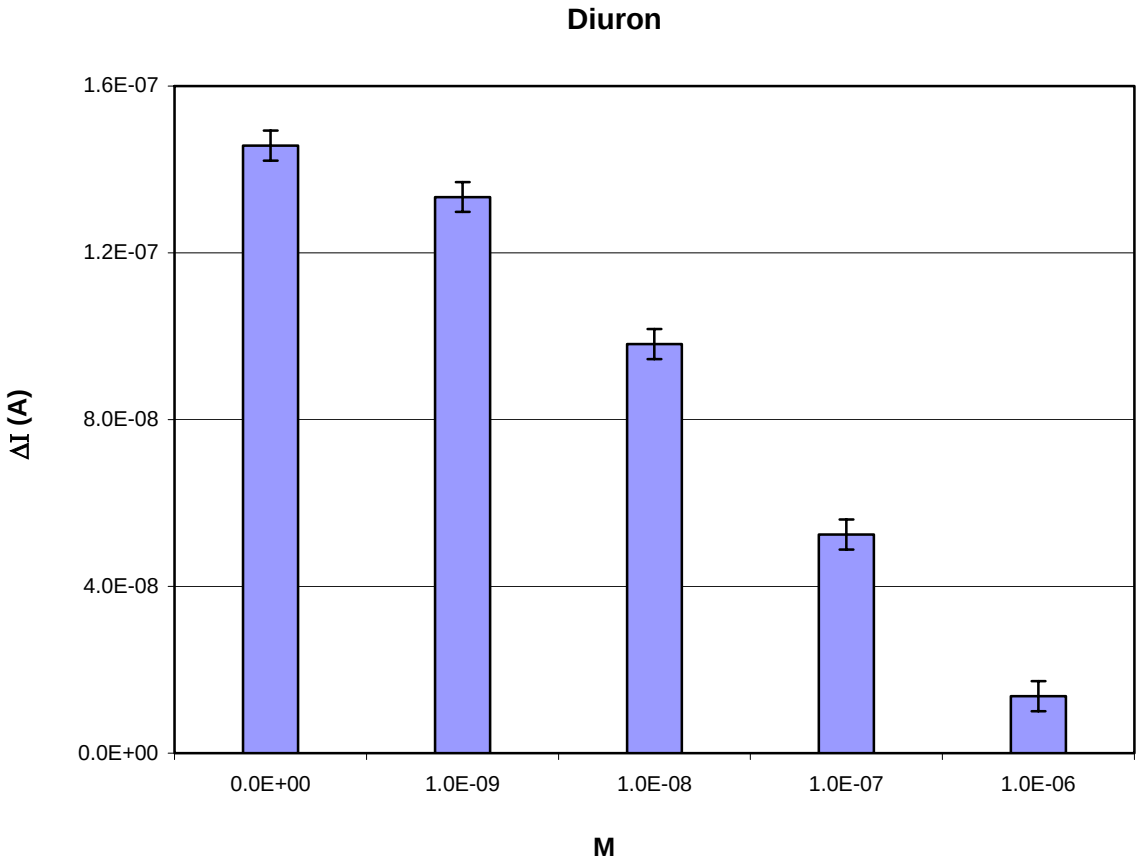


Figure 7.12: Sensor responses (ΔI) for diuron.

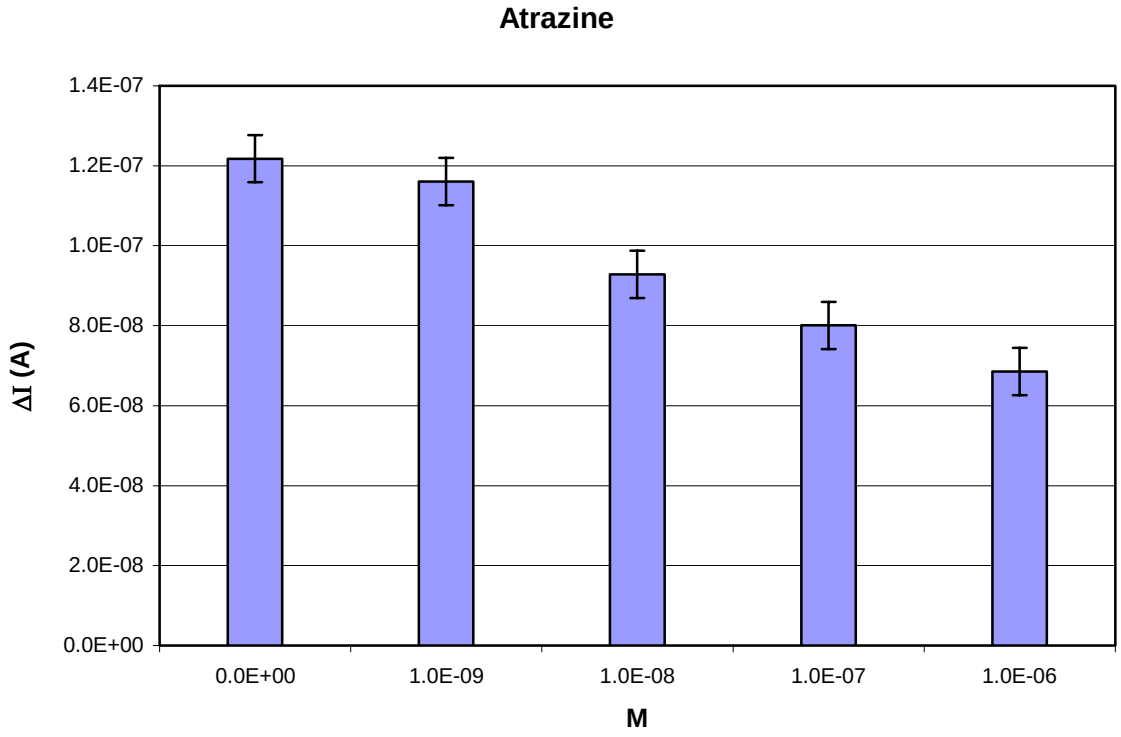


Figure 7.13: Sensor responses (ΔI) for atrazine.

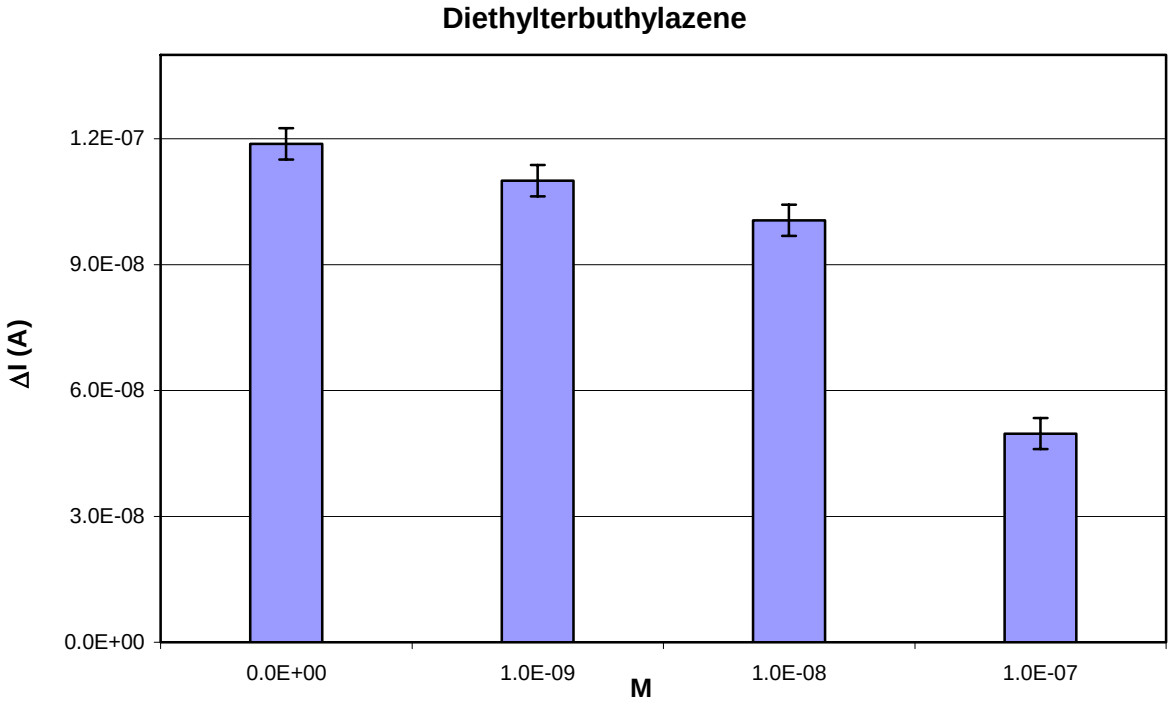


Figure 7.14: Sensor responses (ΔI) for diethylterbuthylazene.

Table 7.2: Results for different herbicides used in Langmuir adsorption equation.

Herbicides	I_{50} (μM)	LOD (nM)	RSD (%)
Atrazine	0.37	9.6	7.8
Diuron	0.98	6.7	2.5
Simazine	0.736	2.1	1.1
Diethylterbuthylazine	0.5	5.3	3.7

The electrochemical signals (magnitude and sensitivity to herbicides) obtained with chloroplasts were similar to these obtained with thylakoids (Touloupakis *et al.*, 2005). In both cases the inhibition of chloroplasts/thylakoids by diethylterbuthylazine was the strongest. In the past it was shown that atrazine has average inhibitory effect on the native thylakoid membranes but that this was smaller than diuron which had shown an almost tenfold larger inhibition effect at these concentrations (Piletskaya *et al.*, 1999). These data were performed in a test system in microtiter plates that were illuminated with a 100 W lamp and the absorbance was measured at 530 nm. In our work, illumination at 650 nm and the close contact of the LED to the working electrode surface was expected to

provide more sensitive data than previous sensors. In all measurements there was not a significant difference between all classes of compounds. However, diuron gave the most reliable signal compared to the triazine compounds. Finally the limit of detection in the nanomole area makes it possible to use the sensor directly in water samples and observe the concentration levels required by EC regulation for waste and drinking water (DWI).

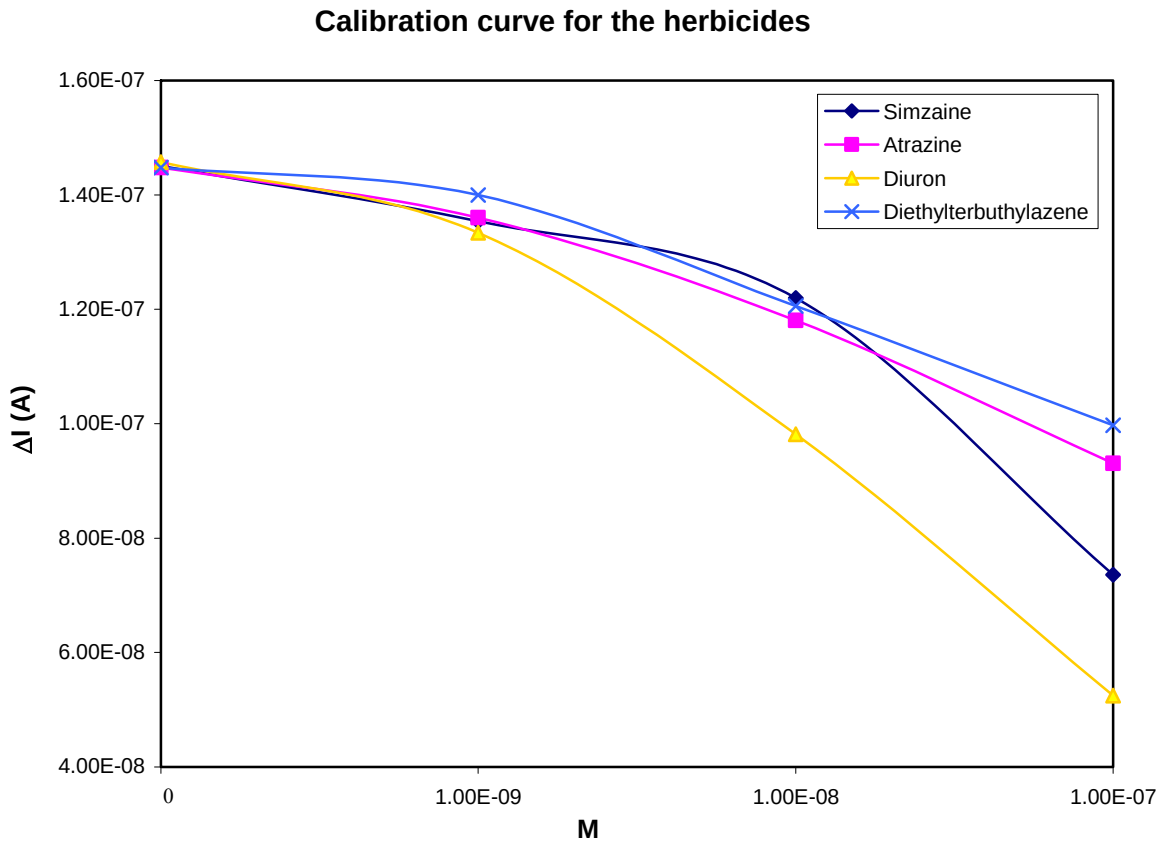


Figure 7.15: Calibration curve for herbicides calculated with Langmuir adsorption coefficient.

7.3.5 Application of biosensor to water samples

The sensor developed in his study has been used for measuring real water samples provided by partners in EU project (Water Research Institute IRSA-CNR). The water samples have been previously analysed using GC/MS and the results were provided and compared with electrochemical data. Several herbicides have been detected in river water samples and the final results are presented in Table 7.3. Since our biosensor was not

designed to give information about individual herbicides but about their total concentration, the correlation of data was made accordingly using total sum of herbicides. The sensor signal was compared with calibration curve for atrazine which represents herbicide with "average" activity. For more specific detection, a separation system would be required such as selective solid phase extraction cartridges. Alternatively we could use mutant organisms with enhanced selectivity developed by other partners in the project (Institute for Plant and Cell Physiology, Martin-Luther Universität, Halle, Germany). This is further discussed in future work.

Filtered river water was passed through a 200 mg Lichrolut EN commercial cartridge (VWR, Germany). Organic compounds were eluted from the cartridge using 5 ml of acetonitrile/methanol (1:1v/v) and then repeated a few minutes later. The extract, after a gentle concentration by N_2 flux into a Turbovap II (Zymark, USA) apparatus, was recovered with ethylacetate at a final volume of 1 ml. The extract obtained was 1000 times concentrated in respect to the environmental sample. The sample was further diluted 3.3 times in Milli-Q water. The concentrations of the herbicides in the concentrated sample are reported in Table 7.3. These samples were used for analysis with chloroplast-based biosensor.

Table 7.3: Results of analysis of Po water samples by GC-MS.

Compound	**Herbicides in river water (μg/l)**	**Concentrated sample (μg/l)**
Diethylterbuthylazene	0.026	7.8
Diethylatrazine	0.027	8.1
Atrazine	0.027	8.1
Terbutylazine	0.106	31.8
Oxadiazon	0.007	2.1
Simazine	0.020	6
Prometryn	0.005	1.5
Sum of herbicides	**0.218**	**65.4**

The preconcentrated sample was diluted (10 times) with a buffer containing 20 mM Tricine, 70 mM sucrose, 15 mM NaCl, 5 mM $MgCl_2$ and 5 x 10^{-5} M CAP at pH 7.8 . The total herbicide concentration was equal to 10^{-8} M. The signal was measured after 5 minutes following 3 flashes. The signal reached a steady plateau at 15 minutes (Figure 7.16). This change in signal (17 nA) was equivalent to the change produced by 5 x 10^{-8}M atrazine (selected as a marker herbicide with "average" activity). The result showed a reasonable correlation between expected and measured figures. It is clear that real quantification would be impossible due to the lack of information about real presence of different types of herbicides which might be more or less active than atrazine. We believe however that the sensor can be used for a fast screening of environmental samples for the presence of herbicides which might be later tested in dedicated environmental laboratories.

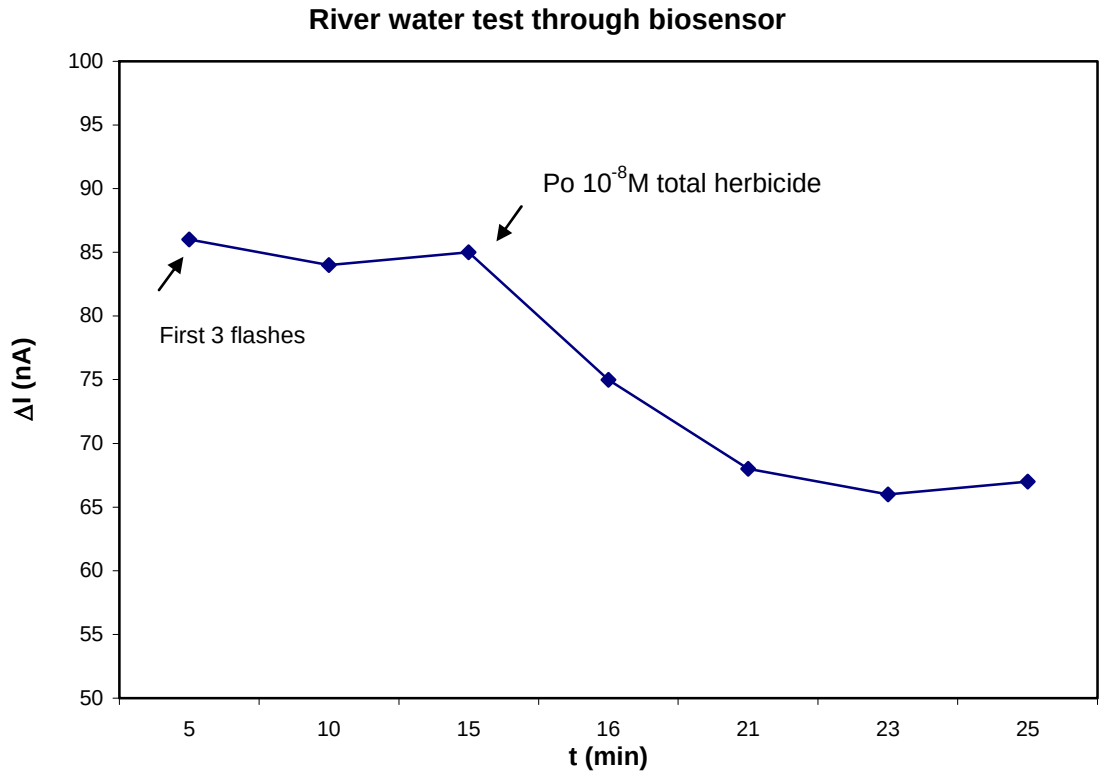


Figure 7.16: Diluted concentration of river water samples run through the biosensor.

7.4 CONCLUSIONS

The immobilisation of chloroplasts by cross-linking with BSA proved to be effective allowing achievement of reproducible and sensitive measurements. The measured lifetime of electrodes stored at – 80 ^{0}C was 3 months. The immobilisation of chloroplasts using BSA/GA gel formation and correct storage temperature (-80^{0}C) permitted preservation of photochemical activity at a good level. The operational lifetime was 3 hours using chloroplasts immobilised on the working surface. Reproducibility of the measurements using different sensors was reasonably high for a crude fabrication procedure (RSD = 9.11%). Furthermore, the required quantity of the material used on the electrode surface was minimal compared to other techniques. The use of the flow cell system should allow automation of the measurements in future work. The advantages over previous biosensors (Loranger and Carpentier, 1994; Rouillon *et al*., 1995) include the higher sensitivity (Figure 7.9) and also the simplicity of the electrode preparation and measurements. Using DCPIP as the mediator permitted decrease of the redox potential and avoidance of potential interference from the environmental samples. In contrast to another mediator – DQ, which also possessed a low redox potential, the solubility of DCPIP in water was significantly higher which allowed the avoidance of the need to use an organic solvent.

The use of the Hill reaction in order to detect herbicides is quite attractive due to the efficiency of the technique. Measurements could be performed simultaneously and would be specific for all inhibiting herbicides present in the sample. The herbicides detected using the Hill reaction were triazines and phenylureas as these are most important herbicides required by EU regulations. In general it was found that all herbicides were measured at concentrations ranging from 10^{-6} M to 10^{-9}M. In conclusion, the biosensor developed in this study offers sensitive detection to a class of photosynthesis-inhibiting herbicides and the possibility of application in analysis of environmental water samples.

CHAPTER 8. FINAL CONCLUSIONS AND FUTURE WORK

8.1 MEETING THE PROJECT OBJECTIVES

The objectives of this thesis were to research and develop a biosensor for electrochemical detection of herbicides that would meet the requirements of the EC project (Framework Programme 5/ Contract QLK3-2001-01629). All these objectives have been met during development of the project.

8.1.1 Components selection and optimisation

Chapter 3. The construction of the sensor and the selection of components were described according to the analytical needs and need for low cost of manufacturing and ability for mass production. Screen-printed electrodes were selected for the project development due to low cost and easy manufacture. Horseradish peroxidase was selected due to its high activity, low price and relatively high stability. The mediator hydroquinone was selected after research on its optimal potential, its cost, stability and low solubility in aqueous solution. The polymer thioacetale was selected on the base of its suitability for fast and effective covalent binding of protein and also on its easy integration with sensor through screen-printing.

Chapter 5. The imprinted polymers have been designed in accordance with previously published results of the development of polymer with selectivity for atrazine.

Chapter 6. The selection of the components for the Hill reaction was literature based. The mediator 2.6 dichlorophenol indophenol was selected after testing two more suitable mediators found from a search of the literature. This mediator provided the optimum balance between the stability and low solubility in aqueous solution. The herbicides tested were chosen as the most common herbicide still posing an environmental threat.

8.1.2 Sensor testing and stability

Chapter 4 describes the design and optimisation of a hydrogen peroxide sensor. In the laboratory, the sensors demonstrated reasonable storage and operational stability (they can be stored for 2 months at 4 ^{0}C and operate continuously for 3 hours) and good reproducibility of measurements (RSD = 5-7%). The lower limit of detection of 0.1 μM has proven the suitability of the sensor for several practical applications. The performance of hydrogen peroxide sensor was found equal or better to the examples previously described in the literature.

Chapter 7 describes a sensitive sensor for herbicide detection. Screen-printed electrodes integrated with spinach chloroplasts have shown a sufficient limit of detection for herbicides such as ureas and triazines (the detection limit was 1-8 nM depending on the type of herbicide). The sensor has been shown to work successfully under different storage conditions. The herbicide sensor had a good operational stability (it worked for up to 3 hours at room temperature) and long shelf-life (it can be stored at -80 ^{0}C for 3 months). An experiment with real water samples showed a good correlation between the data obtained with GC-MS and the chloroplast-based biosensor developed. These properties are enouraging for a development of a practical sensor for environmental monitoring.

8.2 FUTURE WORK

8.2.1 Development of sensor prototype

Before the sensor prototype can be recommended for a practical application more work needs to be done to optimise the immobilisation procedure. This procedure should be more suitable for mass production. The signal from chloroplasts immobilised on the screen-printed electrodes surface could be further enhanced using a different cross-linker such as BS3. More research is also needed to characterise the electrodes and if necessary, to minimise the influence of other parameters such as ambient temperature, storage temperature, and pH. To increase the selectivity of measurements sensor should be integrated with selective extraction protocol. The initial project proposal for the EU project involved using mutant bacteria cell with enhanced selectivity for different

herbicides. It is known that specific amino acid mutations of D1 protein can provide different degrees of sensitivity and resistance to herbicides. Some initial steps in this direction were made by our partners. *PsbA* genes were amplified from different Chlamydomonas mutants and plant species and sequenced by partners. Unfortunately this work has not been completed in time and we did not have access to these materials. Potentially this could be considered as a promising way forward. Future work could also include measurements with bacteria such as rhodobacter spheroids. Preliminary tests under amperometric conditions had shown a promising signal without, however selectivity to triazines herbicides. More classes of biological organisms could be tested.

8.2.2 Other activities

During the project development we identified three promising areas which could be recommended for continuation of scientific and applied research. The first included the development of a hydrogen peroxide sensor. The prototype developed here possessed good properties to recommend it for a development of practical sensor for monitoring of e.g. disinfection procedure in water treatment and in food industry. More optimisation work needs to be done in order to realise this possibility in practice. This second area included the development of screen-printed electrodes containing thyoacetale based polymers. These electrodes can be used effectively for the immobilisation of enzymes, DNA and other biological molecules. The future development of such electrodes might have excellent commercial perspectives. The third area includes the development of an imprinted “sandwich” type sensor. Screen-printing on the surface of MIP membrane might lead to the development of a new type of robust sensors for biotechnology, environment and clinical applications. However to optimise the deposition and operational parameters of such systems more work needs to be carried out.

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